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ADAPTATION TO INCREASED AND DECREASED FUNCTIONAL DEMAND IN RAT SKELETAL MUSCLE DEPLETED OF HIGH ENERGY PHOSPHATES.

BY

DANIEL ROBERT MARSH ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fullfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF PHYSIOLOGY

Edmonton, Alberta
Spring, 1995.

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ABSTRACT

The effects of reducing the intracellular concentration of phosphocreatine and adenosine triphosphate on skeletal muscle properties, protein turnover and adaptation to functional perturbations form the core of this thesis. Ingestion of the creatine analogue β-guanidinopropionic acid (GPA) significantly reduced the concentration of phosphocreatine and adenosine triphosphate in the soleus, plantaris and the tibialis anterior muscles of rats. The subsequent changes in cross-sectional area, succinate dehydrogenase activity and α-glycerophosphate dehydrogenase activity were examined using quantitative histochemical and image analysis techniques. Adaptations in type I and II fibres from the tibialis anterior (fast dorsi flexor), plantaris (fast plantar flexor) and the soleus (slow plantar flexor) muscles were generally muscle and fibre type specific. The tibialis anterior was most susceptible to GPA feeding, exhibiting the only increase in GPD activity as well as fibre type specific atrophy. In contrast, succinate dehydrogenase activity was increased in all muscles and fibre types. The hypertrophic response of type I and II fibres to the increase in functional demand associated with synergist ablation was not limited when combined with continued GPA feeding. However, succinate dehydrogenase activity was decreased to a greater extent and the increase in total succinate dehydrogenase following synergist ablation was attenuated. When tetrodotoxin (TTX)-induced paralysis was superimposed on continued GPA feeding the fibre atrophy, increased percentage of type II fibres and decreased succinate dehydrogenase activity was equal to or greater than the changes in single fibres of the soleus, plantaris, and tibialis anterior from control rats. Therefore, the adaptations associated with GPA feeding and decreased phosphocreatine and adenosine triphosphate concentrations have a neuromuscular activity dependent component. Measurement of protein turnover indicated that GPA feeding increased protein synthesis in the plantaris and tibialis anterior, but had no effect on the soleus. Tetrodotoxin-induced paralysis caused marked atrophy in both control and GPA-fed muscles. In the soleus, atrophy could be attributed to a decreased rate of protein synthesis and not a decreased rate of

degradation. In contrast, atrophy in the predominantly fast-twitch plantaris and tibialis anterior muscles was due to an increased rate of degradation without any change in the rate of synthesis. The response of protein turnover to TTX-induced paralysis was similar in control and GPA-fed rat muscles.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Introduction

Movement is an important characteristic of animals. For vertebrates, movement is a fundamental means of interacting with and adapting to their environment. They rely on movement to obtain food, elude predators and to reproduce. Vertebrate movement is accomplished by contractile tissue highly organized into muscle cells attached to an internal skeleton. Muscle cells are long, cylindrical, multi-nucleated fibres within which contractile proteins are organized into serially arranged sarcomeres. These fibres are packed into fascicles and in turn are grouped within a sheath of connective tissue to form a discrete muscle that attaches to the skeleton to generate force. The nature of force production in muscle is dependent on the cellular composition of contractile proteins, membrane proteins and metabolic enzymes. This highly complex assortment of proteins determine whether the generation of force by a motor unit is fast and easily fatigued, fast and resistant to fatigue or slow and resistant to fatigue. These characteristics led to the classification of motor units and their respective muscle fibres into three distinct types (Burke et al., 1971). In addition, a classification of muscle fibres as type IIb, IIa and type I, respectively, based on myosin stability following alkaline or acidic incubation prior to myofibrillar ATPase staining had been established (Brooke and Kaiser, 1970). More recently, the sophisticated use of myosin antibody staining has led to additional classification of muscle fibre types into IIc, IIx and Ib (Schiaffino et al., 1989). For the purposes of this thesis, muscle fibres will be classified only into the major myosin isoforms of fast and slow myosin heavy chain, type II and I fibre types, respectively.

Movement is a complex task which requires contributions of many skeletal, muscular and neural elements. In mammals, each limb has muscles that vary in size and tension generating characteristics that can be recruited by the nervous system to achieve a wide variety of functions. When environmental perturbations require a change in function, the cellular composition of muscle proteins and the associated morphological, contractile and metabolic properties of muscle fibres also change. The nature of functional adaptation in skeletal muscle is an important biological question in both basic science as well as having many applications for medical rehabilitation from injury or disease.

1.2 Embryological development

Muscle originates from the mesodermic layer of the embryo. In the embryo, an early phase of muscle fibre differentiation occurs whereby myoblasts are committed to three intrinsically different primary myogenic lineages; fast, fast/slow and slow (Miller and Stockdale, 1986). Primary myotubes formed from these myoblasts differ in their myosin heavy chains independent of innervation. However, the maintenance and maturation of the slow myosin heavy chain is dependent on innervation (Gunning and Hardeman, 1991). Secondary myotubes are formed from myoblasts of a single myogenic lineage with fibre diversity and maturation of slow and fast myosin heavy chain dependent on innervation (Miller and Stockdale, 1986; Gunning and Hardeman, 1991). The early phenotype of primary and secondary myotubes in vivo is not predictive of adult fibre phenotype. Rather, there appears to be a crucial developmental decision that determines the adult phenotype. The adult phenotype, at least at the level of myosin heavy chain expression, may reflect the influence of growth factors, hormones and neural factors upon the genetic predisposition of the myoblasts (Gunning and Hardeman, 1991).

Normally, secondary muscle fibres are generated along the walls of the primary myotubes under the basement membrane (Fredette and Landmesser, 1991). In muscles which lie in close proximity to the bone, such as the soleus, the generation of secondary myotubes leads to an intermingled pattern of muscle fibres which express fast or slow myosin heavy chain. In the more superficially placed muscles, secondary myotubes are generated primarily in the more superficial regions of the muscle which then predominantly express fast myosin heavy chains (Condon et al., 1990). Interestingly, these muscle fibres will be under different mechanical stresses than muscle fibres originating from primary myotubes which formed along the bone.

1.3 Molecular biology of skeletal muscle

The central character of skeletal muscle is contractile activity. Therefore, it is not surprising that the components of the contractile apparatus represent the most thoroughly characterized markers of muscle fibre type. The variation in contractile properties of different muscle fibres is due to eight multigene families that contribute the major components of the muscle sarcomere (reviewed in Pette and Vrbova, 1985). These genes are myosin heavy chain, alkali myosin light chain, regulatory myosin light chain, actin, troponin C, troponin I, troponin T, and tropomyosin. All but the actin gene encode proteins that are specific for fast and slow fibres. For instance, myosin heavy chain multigene family encodes 3-5 fast isoforms in addition to the slow isoform that are specific to adult muscle fibres (Gunning and Hardeman, 1991; Pette and Vrbova, 1985). In addition to the contractile apparatus, the Ca^{2+}-ATPase of the sarcoplasmic reticulum is also encoded by a multigene family that generates a slow and fast isoform (Ruegg, 1987). The characteristic contraction times of muscle fibre types, particularly half relaxation time and total contraction time, are attributable to the rate of Ca^{2+} sequestration by the sarcoplasmic reticulum. Metabolic enzymes, such as lactate dehydrogenase, also exist as multiple isoforms of varying activity that are expressed in specific myofibre types of skeletal muscle (Leberer and Pette, 1984).

1.3.1 Gene expression and protein turnover

The regulation of gene expression in muscle occurs at multiple levels. Many muscle specific genes have regulatory regions that control transcription. These regulatory regions have multiple binding sites for a heterogeneous array of *trans*-acting factors which are capable of transcriptional regulation of muscle gene promoters. The levels of mRNA of each protein can be regulated by differences in transcript processing, transport, and/or mRNA stability (Wade and Kedes, 1989). Regulation of muscle proteins also occurs at the level of translational efficiency and protein turnover (Booth and Kirby, 1992). Thyroid hormone has also been shown to influence gene expression in skeletal muscle; the fast myosin isoform is expressed to a greater extent in the soleus and gastrocnemius following an increase in thyroid hormone levels (Lomax and Robertson, 1992). In addition, following thyroidectomy there is an increase in the slow myosin isoform in skeletal muscles of the rat (Ianuzzo et al., 1977). During cardiac hypertrophy the level of thyroid hormone was shown to be directly responsible for increased myosin heavy chain expression and that hypertrophy was secondary to increased cardiac work (Klein and Hong, 1986). Furthermore, changes in fast myosin heavy chain expression following increased or decreased loading of skeletal muscles appear to be sensitive to thyroid hormone levels (Baldwin et al., 1992).

Protein synthesis in muscle involves a cyclic process in which ribosomal subunits associate with mRNA, tRNA, and other factors to form polysomes. When the translation of mRNA is completed, the polypeptide chain, mRNA and ribosomal subunits dissociate. Each of the steps of this ribosome cycle represents a complex sequence of reactions involving several enzymes, protein factors and cofactors. Control of protein synthesis occurs through changes in the rate of peptide-chain initiation or elongation/termination, by the number and distribution of ribosomes, or by the concentration of translatable mRNA. Changes in the capacity for translation and translational efficiency are two general categories by which the rate of protein synthesis can be modulated (Vary and Kimball, 1992).

Protein degradation in skeletal muscle is accomplished by several kinds of proteases. These enzymes are classified into three categories as judged by their optimum pH: alkaline protease, neutral protease activated by Ca^{2+} and acidic protease or cathepsins (Obinata et al., 1981). The localization of the proteases varies from intramuscular mast cells, to the cytoplasm and lysosomes. There is even a remarkable range of substrate specificity between proteases of the same classification. Furthermore, sensitivity to ATP depletion and/or Ca^{2+}-activation also varies between proteases. Increased activity of cathepsins B and D as well as Ca^{2+}-activated proteases have been linked to increased rates of myofibrillar protein degradation in a variety of disuse models (Loughna et al., 1986; Ellis and Nagainis, 1984).

The rate of protein synthesis and degradation are influenced by the hormones insulin, thyroxine and glucocorticoids as well as insulin-like growth factors (IGF-1 and IGF-2) (Sugden and Fuller, 1991; Florini, 1987). The effect of insulin on protein turnover in vivo is complex due to its effect on the plasma concentrations of other hormones and branched-chain amino acids. It is believed that insulin increases the fractional rate of protein synthesis at the level of translation, but the increase is dependent on adequate levels of amino acids (Sugden and Fuller, 1991). In a manner similar to insulin, IGFs are reported to have an anabolic effect on skeletal muscle by increasing protein synthesis and decreasing protein degradation (Florini, 1987). Decreased levels of insulin stimulate an increase in glucocorticoids. Glucocorticoids decrease the rate of protein synthesis and reduce muscle's capacity for protein synthesis (Sugden and Fuller, 1991). The degree to which glucocorticoids affect protein turnover may be muscle specific and differ between slow-twitch and fast-twitch muscles (Kelly and McGrath, 1986). The muscle atrophy which occurs following chronic glucocorticoid treatment is fibre-type and muscle specific; a more pronounced atrophy of fast-twitch, compared to slow-twitch fibres, occurs in the gastrocnemius to decrease muscle mass to a greater extent than in the soleus (Gardiner et al., 1980). In contrast, a pre-translational action of thyroid hormone altering RNA turnover is suggested to increase muscle's capacity for protein synthesis (Sugden and Fuller, 1991). However, the increase in the fractional rate of protein synthesis of skeletal muscle following increased thyroid hormone did not alter muscle growth due to a concomitant increase in the rate of protein degradation (Carter et al., 1982).

Protein synthesis and degradation are also energy-dependent processes. During peptide chain initiation ATP and GTP are hydrolysed in order to form the large preinitiation complex and the initiation complex (Sugden and Fuller, 1991). The adenylate energy charge ([ATP + ½ADP]/[ATP + ADP + AMP]) has been proposed to regulate the rate of ternary initiation complex formation by influencing GTP regeneration (Walton and Gill, 1976). When the ATP content of in vitro cellular preparations is decreased by metabolic poisoning, the rates of both protein synthesis and degradation are altered. A moderate decrease in ATP concentration either stimulated protein degradation or had little effect, whereas protein synthesis was much more sensitive and decreased substantially (St. John and Goldberg, 1978; Gronostajski et al., 1985; Fagan et al., 1992). In contrast, a drastic decrease in ATP inhibited both rates of protein degradation and synthesis (St. John and Goldberg, 1978; Gronostajski et al., 1985). Furthermore, the rate of protein synthesis in an in vitro ATP-generating system, has been shown to decrease with a decrease in the ATP:ADP or GTP:GDP ratio (Hucul et al., 1984). Recently, Fagan et al. (1992), demonstrated that ATP depletion either decreased in vitro protein degradation or, in the presence of Ca^{2+}, increased protein degradation. The effect of a depletion of ATP concentration on in vivo protein turnover remains to be determined.

1.4 Mechanical factors which alter phenotype

Within developmental and genetic restraints, a muscle fibre's complement of contractile proteins and enzymes can change in response to environmental influences. Various experimental models have been used to examine the remarkable adaptive capacity of muscle. Experiments using denervation, cross-reinnervation of fast muscles with slow nerves (and vice versa), chronic electrical stimulation, and neurotoxin-induced paralysis demonstrated the importance of neural activation and nerve trophic factors in the maintenance and determination of mature muscle properties (reviewed in Pette and Vrbova, 1985). However, the extent of neural control of muscle properties is not complete (Edgerton et al., 1985). Other experiments using models of stretch, immobilization, hindlimb unweighting, and synergist ablation demonstrated that mechanical factors also contribute to the maintenance and determination of muscle properties (reviewed in Roy et al., 1991). Specific protein systems or fibre properties may be more susceptible to particular external influences. Neurotrophic factors are acknowledged to primarily determine protein expression within the sarcolemma and regulate its properties (Bray et al., 1979). The activation and development of force in a muscle fibre are essential factors in maintaining and/or increasing fibre size (Roy et al., 1991). The concentration of mitochondrial enzymes and the speed of calcium regulation is determined largely by activity patterns and the subsequent metabolic demands of the fibre (Pette and Vrbova, 1985). Although a system may be sensitive to one form of activation, this does not imply that other factors do not contribute to its regulation. Instead, muscle properties are regulated by a balance of contributing factors that influence gene expression and protein turnover (see above).

Gene expression of contractile and metabolic proteins may be regulated in a coordinated fashion. For instance, there is a strong interdependence in the regulation of myosin heavy chain and glycolytic enzymes (Roy et al., 1991). Furthermore, a significant number of glycolytic enzymes are bound to contractile proteins and form a glycolytic complex (Brooks and Storey, 1988). It is possible that there is a functional advantage to match the rate of ATP utilization by myosin ATPase and the rate of ATP supply from glycolytic or glycogenolytic activity. Since fast myosin can hydrolyse ATP at twice the rate of slow myosin it seems logical that the expression of proteins associated with myosin type and a fibre's glycolytic potential are closely linked (Roy et al, 1991).

1.5 Models of decreased and increased contractile activity

1.5.1 Tetrodotoxin-induced paralysis

Tetrodotoxin (TTX) is one of the most toxic substances known. The toxin has been observed only in amphibians of the family Salamandridae and in fishes of the suborder Tetraodontoidae and has been isolated from the California newt, *Taricha torosa*, and the Japanese puffer fish, *Sphoeroides rubripes*, for scientific analyses (Mosher et al., 1964). The toxin specifically affects the sodium channels of muscle and nerve rendering them impermeable to Na^+ and Li^+ ions (Hille, 1968). The muscular inactivity created by TTX-induced paralysis differs significantly from that of denervation. Neural application of TTX does not block fast axonal transport or cause nerve degeneration (Czéh et al., 1978; Bray et al., 1979). Although TTX-induced paralysis caused changes in resting membrane potential and acetylcholine receptor density of muscle, the magnitude of change was less than that of denervation (Bray et al., 1979). Paralysis induced by TTX also had less effect on Ca^{2+} ion transport and Ca^{2+}-ATPase activity of the sarcoplasmic reticulum (Wan and Boegman, 1981a) and had less effect on the activity of lactate dehydrogenase, creatine kinase and pyruvate kinase than denervation (Wan and Boegman, 1981b). These early studies of the effects of tetrodotoxin were either acute, 3-6 days, and used a single application of TTX in a prepared cuff or capillary which was then placed on the nerve (Czéh et al., 1978; Bray et al., 1979), or chronic which relied upon repeated neural injections of TTX to maintain paralysis over days and weeks (Wan and Boegman, 1981a, 1981b). In contrast to the acute studies, the chronic studies of Wan and Boegman (1981a, 1981b) reported no satisfactory method of proving the maintenance of TTX-induced paralysis, thus their results are unequivocal.

A vast improvement in the chronic application of TTX was realized when osmotic pumps, produced by Alza Corp., were first used to perfuse a nerve (Betz et al., 1980). The use of an osmotic pump, as a method to administer TTX via a catheter and nerve cuff to the sciatic nerve was further advanced by

Spector (1985a,b) and St. Pierre and Gardiner (1985). The absence of the toe-spreading reflex was used to confirm paralysis after surgical implantation. The effectiveness of TTX-induced paralysis was verified by stimulating the nerve proximal and distal to the cuff as well as measuring in vivo EMG responses during locomotion. In each case, a good agreement between the subjective (toe-spreading reflex) and objective (EMG) measurements of paralysis was found. The gait pattern of paralysed animals was characterized by an absence of plantar flexion and the accentuated use of hip flexors to bring the limb forward (St. Pierre and Gardiner, 1985).

With the use of this method, the chronic effects of TTX on a variety of muscle properties have been established. The soleus exhibited significant atrophy of muscle mass and fibre cross-sectional area after 2 and 4 weeks of TTX-induced paralysis (Spector, 1985a). Changes in cross-sectional area did not differ between type I and II fibres and the observed atrophy was less than that which occurred as a result of denervation (Spector, 1985b). The percent composition of type II fibres in the soleus increased by 10% and 20% following 2 and 4 weeks of paralysis, respectively. Consistent with increased type II fibre composition, the rate of tension development and the maximal rate of shortening (Vmax) increased following 4 weeks of TTX-induced paralysis. There was no difference in the changes in soleus fibre type composition and contractile parameters induced by TTX and denervation (Spector, 1985b). No report on the metabolic changes of the soleus to TTX-induced denervation was found.

The predominantly fast-twitch gastrocnemius muscle was similarly affected by TTX-induced paralysis. Muscle wet weight and fibre cross-sectional area were decreased by 37% and 59%, respectively, following 2 weeks of TTX. The degree of fibre atrophy was similar between type I and II fibres. The total protein and stromal protein concentration of the plantaris was unaltered, but there was a preferential loss of myofibrillar protein following TTX-induced paralysis (St. Pierre and Gardiner, 1985; Gardiner et al., 1992). The maximum tetanic tension generated per gram of muscle was decreased by 38%, whereas, the rate of tetanic tension development increased following TTX-induced paralysis (St. Pierre and Gardiner, 1985). The half relaxation time and contraction time also increased following TTX, suggesting a decreased Ca^{2+} uptake by the sarcoplasmic reticulum (MacIntosh et al., 1988). Metabolic properties of gastrocnemius were also altered by TTX as the enzyme activity of citrate synthase, phosphofructokinase and α-glycerophosphate dehydrogenase were all decreased (St. Pierre et al., 1988). The succinate dehydrogenase activity was also decreased in type I and II fibres of the gastrocnemius (Gardiner et al., 1992).

Investigations employing TTX-induced denervation are limited in number. Spector (1985a,b) has examined the morphological and contractile properties of the soleus following 2 and 4 weeks of TTX, but no examination of the metabolic properties of the soleus has been undertaken. Gardiner and associates have been the most active in using TTX-denervation, but their work has concentrated on the gastrocnemius (St. Pierre and Gardiner, 1985; St. Pierre et al., 1988; MacIntosh et al., 1988; Gardiner et al., 1992). Thus, there is a need for continued work examining muscle properties of the soleus, plantaris and fast-twitch extensors following TTX-induced denervation.

1.5.2 Synergist ablation

The increase in muscle activity which occurs when a muscle's synergists are surgically removed has been used as an experimental paradigm to investigate muscle adaptation to increased functional demand. Although the technique has been used for the tibialis anterior and gastrocnemius, it is most successful when used for the extensor digitorum longus, soleus or plantaris muscles which do not make up the majority of their muscle group mass. The surgical removal of the gastrocnemius and soleus from the triceps surae muscle group increases the recruitment level and subsequent EMG amplitude of the plantaris without altering its temporal pattern of activation (Gardiner et al., 1986; Roy et al., 1991). The increased functional demand is sufficient to stimulate a hypertrophic response of both type I and II fibres. It has been proposed that muscle fibre damage resulting from the increased functional demand induces satellite cell proliferation. Myoblasts derived from mitotically active satellite cells either fuse with their associated growing muscle fibre, thereby maintaining a constant myonuclear:sarcoplasmic volume ratio, or generate muscle fibres de novo (Darr and Schultz, 1987). A recent study using gamma irradiation of overloaded mouse muscle showed that satellite cell proliferation was a prerequisite of muscle hypertrophy induced by synergist ablation, but was not required for maintenance, or alteration of, myosin heavy chain phenotype expression (Rosenblatt and Parrry, 1992).

In addition to fibre hypertrophy in the plantaris, a shift in metabolic and contractile properties towards slow-twitch muscle properties occurs. For instance, the percent composition of type I fibres was increased by 10-20% and glycogenolytic and glycolytic enzyme activity was decreased by 40% (Ianuzzo and Chen, 1979; Baldwin et al., 1982). The time to peak tension, half relaxation time and fatigue resistance increase, whereas Vmax is significantly decreased (Roy et al., 1982). An examination of plantaris motor units following synergist ablation indicated that tetanic tension increased by the same relative magnitude in all motor units. In addition, the proportion of motor unit types was significantly altered. Higher proportions of fast fatiguable and slow motor units and lower proportions of fast fatigue-resistant and intermediate fatigue-resistant motor units were found in the overloaded plantaris relative to control (Olha et al., 1988). The activities of tricarboxylic acid cycle enzymes succinate dehydrogenase and malate dehydrogenase are either reduced or unchanged (Ianuzzo and Chen, 1979; Baldwin et al., 1982). The rate of oxidation of pyruvate and palmitate was also unchanged, but ketone oxidation was increased following synergist ablation (Baldwin et al., 1981).

1.6 Putative mechanisms of adaptation in skeletal muscle

It is unlikely each factor contributing to the maintenance of muscle fibre phenotype has its own mechanism. Instead, a common denominator of regulatory factors likely exists through which the effects of individual factors are modulated.

1.6.1 Mechanical stretch

Weight bearing, contractile activity, chronic electrical stimulation, synergist ablation and paralysis all alter the degree of mechanical stretch on muscle fibres. Putative transducers of mechanical forces fall into two general categories; extracellular matrix molecules and plasma membrane associated molecules (Vandenburgh, 1992). Many extracellular matrix molecules bind to specific receptors in the plasma membrane that are part of the integrin receptor superfamily (Buck and Horwitz, 1987). These receptors, in turn, are linked to the cytoskeleton that regulates cell shape and may play an active role in regulating mechanically induced cell growth Vandenburgh, 1992). Stretch-induced alterations in plasma membrane associated molecules such as Na^+-K^+-ATPase, ion channels, phospholipases, G proteins and their associated cytoplasmic second messengers including prostaglandins, cAMP, inositol phosphates, intracellular Ca^{2+}, and protein kinase C are other possible mechanisms by which cells respond to stretch.
(Vandenburgh, 1992). Vandenburgh (1992) suggests that mechanical forces and growth factors, either exogenous or endogenous, interact synergistically in muscle to generate second messenger cascades which lead to cell growth.

1.6.2 Adenylate phosphorylation potential

Another proposed common denominator of physical factors regulating muscle phenotype is the adenylate phosphorylation potential. Meerson (1975) proposes that chronic depletion of ATP and accumulation of ADP and AMP following activity stimulate mitochondrial biogenesis and the synthesis of structural proteins. The increased mitochondrial density equilibrates the rate of ATP synthesis with the rate of ATP utilization imposed by the increased functional demand. The increased synthesis of structural and contractile proteins further matches cellular capacity with functional demands. Therefore, Meerson (1975) proposes that a feedback mechanism exists which closely regulates ATP concentration in the cell via biogenesis of mitochondria and synthesis of contractile proteins. In addition, as discussed previously (section 1.3.1) ATP is also a potentially rate limiting substrate and/or modulator of protein synthesis.

The expression of enzyme isoforms can be optimized for the conservation of ATP, whereby the rate of ATP hydrolysis by the various ATPases is decreased. The energy demands of postural muscles composed primarily of type I fibres is higher than that of muscles composed predominantly of type II fibres. The difference in energy demand is due largely to activity patterns. Type I motor units of the rat soleus are active 22-35% of the time over 24 hours compared to 1.6-5% and 0.04-0.22% for type IIb and type IIa motor units (Hennig and Lomo, 1985). The energy costs of the postural soleus is matched by the phenotypic expression of high levels of oxidative enzymes and the more energetically efficient slow myosin heavy chain isoform (Goldspink et al., 1970; Crow and Kushmerick, 1982). When functional demand is increased, such as in chronic electrical stimulation or synergist ablation, levels of oxidative enzymes in muscle are increased as well as the expression of slow myosin heavy chain, thus optimizing energy conservation (Pette and Vrbova, 1985; Roy et al., 1991). Indeed, it has recently been demonstrated that in soleus and extensor digitorum longus muscles of the mouse, energy cost to maintain tension is decreased following a fast-to-slow transition of myosin isoforms (Moerland and Kushmerick, 1994). The transition of myosin isoforms was due to a chronic diet of the creatine analogue ß-guanidinopropionic acid.

1.7 ß-guanidinopropionic acid

Creatine is synthesized in the liver and kidney, but not in muscle. Therefore it must be transported via the bloodstream to skeletal muscle. A specific, Na^+-dependent, integral membrane protein carrier system transports creatine from the plasma into the muscle cell against a concentration gradient

(Loike et al., 1988). Once inside the muscle, creatine is phosphorylated and stored as the high energy phosphate, phosphocreatine (PCr). The creatine analogue ß-guanidinopropionic acid (GPA) competes with creatine for transportation into the cell where it too is phosphorylated. However, it will accumulate inside the muscle cell as PCr is hydrolysed because of its twofold higher K_m and slower maximal rate of reaction ($V_{max} < 1/300$) with creatine kinase compared with that of creatine (Chevli and Fitch, 1979). Petrofsky and Fitch (1980) suggested possible toxic effects of GPA feeding on skeletal muscle should be considered. To date there is no evidence of toxic effects of GPA feeding on skeletal muscle. Moerland et al. (1989) observed no changes in daily activity and no significant alteration in thyroid hormone levels in mice. There also was no change in wet to dry weight ratio of muscle (Fitch et al., 1974), but rats fed GPA do exhibit a period of growth retardation (Fitch et al., 1978).

In normal muscle, the relatively small cellular pools of ATP (2-5 mM) are continuously and efficiently replenished from the larger pools of PCr (20-35 mM) through the reaction catalyzed by creatine kinase:

$MgADP^- + PCr^{2-} + H^+ \leftrightarrow MgATP^{2-} + creatine$

Over a period of weeks, GPA feeding progressively decreases PCr concentration by as much as 90%. Presumably, the depleted pool of PCr is no longer capable of buffering the cellular concentration of ATP and consequently ATP concentration is reduced by 50% (Fitch et al., 1974, 1975). Alternatively, muscle ATP content has been suggested to be inversely related to the muscle's fatigue resistance (Goldspink et al., 1970) and GPA feeding greatly increases muscle fatigue resistance (Petrofsky and Fitch, 1980). The resting levels of ADP and inorganic phosphate (P_i) were not altered by GPA feeding in the tibialis anterior (Fitch et al., 1975), but resting [ADP] was decreased by 25% in the gastrocnemius (Fitch et al., 1974).

The altered resting levels of ATP, PCr and ADP are manifested in changes of muscle metabolism. During muscle contraction induced by electrical stimulation, GPA-fed muscles lack the glycogenolytic/glycolytic stimulus that the release of P_i from the hydrolysis of PCr provides (Meyer and Kushmerick, 1986). An impairment in glycolysis was apparent as the accumulation of P_i and lactate and the depletion of glycogen was significantly less than in control muscle (Fitch et al., 1975; Meyer et al., 1986). The activity of phosphorylase, phosphofructokinase and lactate dehydrogenase are decreased in the gastrocnemius, plantaris and soleus muscles following GPA feeding (Shoubridge et al., 1985). In contrast, glucose transport into muscle and its phosphorylation by hexokinase is increased following GPA feeding (Ren et al., 1993). Due to the decrease in glycolytic enzyme activity and increased glucose transport the glycogen content of the gastrocnemius and plantaris increased by 185% following GPA feeding (Shoubridge et al., 1985). The activity of AMP deaminase, which catalyzes the first step of the purine nucleotide cycle, is greatly decreased in skeletal muscle following GPA feeding. However, despite the decreased AMP deaminase activity, IMP production during electrical stimulation was not altered (Ren and Holloszy, 1992).

In contrast to glycolytic metabolism, many aspects of oxidative metabolism are increased following GPA feeding. Although mitochondria isolated from GPA-fed rats and control rats are qualitatively similar with respect to specific enzyme activities, respiratory rate and degree of coupling, mitochondrial content is increased by 30-40% following GPA feeding (Shoubridge et al., 1985). In conjunction with the increased mitochondrial content, the activities of oxoglutarate dehydrogenase, hydroxyacyl-CoA dehydrogenase and citrate synthase were increased by 30-40% in the fast-twitch plantaris and gastrocnemius muscles (Shoubridge et al., 1985). Shoubridge et al. (1985) reported no change in oxidative enzyme activity of the soleus.

It is unclear if the decreased levels of PCr and ATP and alterations in muscle metabolism following GPA feeding alters the hypothetical "triggering" response of adenylate phosphorylation potential to changes in functional demand. After intense electrical stimulation (3 contractions/sec for 2 min) under anoxic conditions, the ATP concentration and the adenylate phosphorylation potential were decreased by similar amounts in control and GPA-fed tibialis anterior muscles (Fitch et al., 1975). Likewise, Meyer et al. (1986) reported no difference in ATP depletion following 150 s of stimulation at 5 Hz between control and GPA-fed gastrocnemius muscles. In contrast, isometric tetanic contractions of either 3, or 1 s duration every 120 or 90 s reduced ATP concentration by 36% and 27%, respectively, in the GPA-fed gastrocnemius-plantaris while no significant decreases occurred in control muscle (Shoubridge and Radda, 1987). However, Meyer et al. (1986) reported no ATP depletion following 1 Hz supramaximal stimulation for 200 s in either control or GPA-fed gastrocnemius. The conflicting reports of the degree of ATP depletion following moderate electrical stimulation makes any conclusion regarding changes in adenylate phosphorylation potential in response to functional demand in GPA-fed muscle tenuous at best.

In addition to the metabolic changes following GPA feeding, changes also occur in contractile properties and muscle morphology. It has been reported (Shoubridge et al. 1985; Shields et al. 1975), that the response of muscle mass and fibre cross-sectional area to GPA feeding varies across muscles and fibre types. For instance, wet weight is reduced in the predominantly fast-twitch plantaris by 30% (Petrofsky and Fitch, 1975; Shoubridge et al., 1985), whereas the wet weight of the soleus is not altered following GPA feeding (Petrofsky and Fitch, Lai and Booth, 1991). Shoubridge et al. (1985) reported that the largest change in relative muscle mass occurred in a muscle with the greatest proportion of Type IIb fibres. Shields et al. (1975) measured the cross-sectional area of fibres from the deep and superficial portions of the gastrocnemius. They reported that the deep, predominantly type I region, showed no changes in cross-sectional area, whereas the type II fibres of the superficial region were decreased in cross-sectional area by two-fold. The fibre type composition of muscle is also reported to be altered following GPA feeding. Moerland et al. (1989) reported that intermediate myosin (FM_2) of the mouse soleus was reduced by 50% and fast isomyosin (FM_3) in the extensor digitorum longus was reduced by 60%. In the rat, GPA feeding increased the proportion of type I fibres in the soleus from 82% to 100% (Shoubridge et al., 1985). Consistent with this increased expression of slow myosin, Vmax and twitch amplitude was decreased and fatigue resistance increased while twitch rise time and half relaxation time and fatigue resistance increased in the soleus following GPA feeding (Petrofsky and Fitch, 1980). There were no changes in contractile parameters of the plantaris following GPA feeding (Petrofsky and Fitch, 1980). The effect of GPA feeding on the morphology or contractile parameters of rat fast-twitch extensors, such as the tibialis anterior or extensor digitorum longus, has not been investigated.

1.8 Objectives

1.8.1 Objective A

To examine the metabolic and morphological properties of single muscle fibres following GPA feeding in muscles that vary in functional as well as metabolic and contractile properties. In Chapter II, individual muscle fibres from the soleus (predominantly type I ankle plantar flexor), plantaris (predominantly type II ankle plantar flexor), and tibialis anterior (ankle dorsiflexor with a mixed type I and II deep region and a type II superficial region) were examined.

Previous studies have compared adaptations between whole muscles, or portions of muscle, that are predominantly comprised of one fibre type to make conclusions concerning fibre-type specific adaptation to GPA feeding (Shoubridge et al., 1985; Shields et al., 1980). By examining the response of single fibres from the soleus, plantaris and tibialis anterior to GPA feeding direct conclusions regarding muscle and fibre-type specific adaptations may be suggested.

Hypothesis

1. Adaptation of single fibres to GPA feeding is muscle and fibre-type specific such that changes will be more pronounced in muscles composed predominantly of type II fibres.

1.8.2 Objective B

To assess the importance of decreased resting ATP and PCr concentration during adaptation to increased (synergist ablation; Chapter III) and decreased (TTX-induced paralysis; Chapter IV) neuromuscular activity. The response of muscle from GPA-fed rats will be compared to muscle from control rats undergoing adaptation to increased and decreased functional demand.

Meerson (1975) and Green et al. (1992) have postulated that the adenylate phosphorylation potential plays an important role in mediating or triggering adaptation to altered functional demand. However, this putative mechanism of adaptation has not been tested. The GPA-induced depletion of PCr and ATP and the subsequent alteration in adenylate phosphorylation potential provides an opportunity to test the importance of cell energy status during adaptation to synergist ablation (Chapter III) and TTX-induced paralysis (Chapter IV).

Hypothesis

2. The GPA-induced depletion of PCr and ATP attenuates adaptation to synergist ablation and TTX-induced paralysis.

1.8.3 Objective C

To investigate the effect of TTX-induced paralysis on adenine nucleotide concentrations and metabolic and morphological properties of single muscle fibres in the tibialis anterior, plantaris and soleus.

Previous studies which have used TTX-induced paralysis to decrease neuromuscular activity have predominately examined the soleus (Spector, 1985a,b) or the gastrocnemius (St. Pierre and Gardiner, 1985; St. Pierre et al., 1987). No known study has compared single fibre properties between a fast dorsiflexor (tibialis anterior), a fast plantar flexor (plantaris) and a slow plantar flexor (soleus) to determine muscle or fibre-type specific responses to TTX-induced paralysis (Chapter IV). These muscles not only differ in function but also in recruitment and activation during gait and stance. The postural muscles soleus and plantaris are activated more frequently than the tibialis anterior. These differences in combination with fibre type percent composition and respective recruitment of slow vs. fast motor units may result in varied adaptation to neuromuscular paralysis.

Hypothesis

3. Muscle and fibre-type specific adaptation to TTX-induced paralysis occurs based on activation and recruitment patterns of muscles and fibre types. The postural fast-twitch muscle soleus will be affected to the greatest degree and the fast-twitch dorsiflexor tibialis anterior to the least degree.

1.8.4 Objective D

To determine if GPA feeding can pharmacologically decrease ATP and PCr and subsequently induce morphological and metabolic adaptations in muscle independent of neuromuscular activity (Chapter IV).

Lai and Booth (1991) have suggested that an "exercise signal" is required in combination with GPA feeding in order for morphological and metabolic adaptations to occur. Eliminating neuromuscular activity with TTX-induced paralysis during continued GPA feeding (Chapter IV) will determine whether the pharmacological depletion of ATP and PCr is sufficient to produce adaptations.

Hypothesis

4. Neuromuscular activity is required for GPA feeding to alter muscle properties towards slow-twitch oxidative metabolism.

1.8.5 Objective E

To determine whether the GPA-induced decrease in PCr and ATP concentration alters in vivo protein turnover in the soleus, plantaris and tibialis anterior muscles.

To examine the impact of TTX-induced paralysis on in vivo protein turnover in normal and GPA-fed soleus, plantaris and tibialis anterior muscles.

Previous studies of in vitro protein synthesis have demonstrated a relationship between rates of protein synthesis and adenine nucleotide ratios as well as absolute levels of ATP (Hucul et al., 1984; Gronostajski et al., 1985). Furthermore, the rate of protein degradation has been shown to be dependent on ATP concentration and intracellular levels of Ca^{2+} (Fagan et al., 1991). However, biochemical poisoning was used to drastically decrease the in vitro levels of ATP and it is not known what other effects this may have on protein turnover. In vivo ATP levels rarely decrease to less than 50% of resting values, therefore, the radical decrease in ATP achieved with biochemical poisons may not be physiological. It is also not known if in vitro protein turnover data from cultured non-muscle cells can be validly extrapolated to in vivo protein turnover measured in skeletal muscle. Thus, the chronic decrease of resting ATP levels achieved by GPA feeding is a useful experimental tool to examine in vivo protein turnover in skeletal muscle (Chapter V).

Hypotheses

5. The rate of protein synthesis is decreased in GPA-fed muscles.
6. GPA feeding increases the rate of protein degradation following TTX-induced paralysis.

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CHAPTER II

Properties of single muscle fibres following chronic depletion of phosphocreatine and adenosine triphosphate in rats fed ß-guanidinopropionic acid.[1]

2.1 Introduction

The creatine analogue ß-guanidinopropionic acid (GPA) has been shown to deplete phosphocreatine (PCr) and adenosine triphosphate (ATP) in muscles by 90% and 50%, respectively (Fitch et al., 1974, 1975). In rats fed a 1 - 2% GPA diet over 6 - 10 weeks, the depressed concentrations of PCr and ATP were associated with altered metabolic and contractile properties in a variety of muscles (Shoubridge et al, 1985; Moerland et al, 1989; Lai and Booth, 1991). Although the decreases in PCr and ATP content are reported to be similar between muscles, the changes in metabolic and/or contractile properties differ across muscles of varying fibre type (Shoubridge et al, 1985; Petrofsky and Fitch, 1980) or mitochondrial density (Lai and Booth, 1990). The mechanism(s) responsible for the metabolic and contractile adaptations observed subsequent to GPA feeding are not known. However, the chronic depletion of PCr and ATP presents an opportunity to examine a possible link between cell energy status and muscle morphologic and metabolic properties. Furthermore, GPA feeding could be used in combination with other experimental perturbations to examine the relationship between [PCr], [ATP] and adaptation. Prior to developing additional experimental strategies, it was necessary to characterize the effect of GPA feeding on additional muscle metabolites as well as the properties of single muscle fibres. Previous studies have compared adaptations between whole muscles, or portions of muscle, that are predominantly comprised of one fibre type. These investigations are limited to indirect conclusions concerning fibre-type specific adaptation.

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[1] Co-authors for this chapter: T. P. Martin - discussion and editing of manuscript; K. D. Strynadka - HPLC technical assistance.

Therefore, the purpose of the present study was to examine the metabolic and morphological properties of single muscle fibres which express either slow or fast myosin heavy chain following GPA feeding in muscles that vary in functional, metabolic and contractile properties. Individual muscle fibres from the soleus (predominantly type I ankle plantar flexor), plantaris (predominantly type II ankle plantar flexor), and tibialis anterior (ankle dorsiflexor with a mixed type I and II deep region and a type II superficial region) were examined with the hypothesis that adaptation of single fibres to GPA feeding would be muscle and fibre type specific.

2.2 Methods

Animals: Female Sprague-Dawley rats (mean 200 g) were singly housed in a light and temperature controlled environment (12h light/dark, 24°C) in accordance with the guidelines of the Canadian Council on Animal Care. Rats were weight-matched prior to pair feeding and randomly assigned to two groups; control (C) and 2% GPA diet (n=8/group). GPA was synthesized by the method of Rowley et al (1971) and then the crystals were powdered and mixed dry with ground rat chow. Pair feeding was based on the weight of food consumed per day by the GPA animals.

Tissue Preparation: Following eight weeks of feeding, GPA and C rats were anaesthetized with sodium pentobarbital (60 mg/kg). The tibialis anterior, plantaris and soleus muscles were removed from the left hind limb, and immediately frozen in liquid nitrogen-chilled isopentane for HPLC analysis. During the dissection, specific care was taken not to disrupt blood flow to each muscle until frozen. Muscles from the right hind limb were then removed, blotted dry and weighed. The distal and proximal ends of the muscle were removed and the mid-belly was mounted on cork and frozen in isopentane cooled to near-freezing with liquid nitrogen. This portion of muscle was later sectioned and used for quantitative histochemical analyses. All other muscles were removed from the limb and the length of each tibia was then measured with a micrometer. While still anaesthetized, animals were euthanized by cervical dislocation.

Phosphocreatine and nucleotide estimation: The high performance liquid chromatographic method of Stocchi et al. (1987) was used to estimate the concentrations of phosphocreatine (PCr), adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), guanidine triphosphate (GTP) and inosine monophosphate (IMP) following an extraction (6% perchloric acid) and neutralization (6M KOH) procedure. Analyses were performed on a 50 μl injection volume using a 3 μm Supelcosil LC-18t column (25 cm x 4.6 mm i.d.) protected with a 5 μm Supelcosil LC-18 guard column (2 cm x 4.6 mm i.d.). Due to the similar size and charge of phosphorylated GPA and PCr, chromatographic separation of individual peaks in the GPA-fed muscle was not possible. Therefore, PCr was estimated spectrophotometrically (Harris et al. 1974). There was no difference in the estimation of PCr determined spectrophotometrically or by HPLC in the control muscles.

Histochemical Analyses: The muscle samples were cut into 10 μm thick serial sections with a cryostat (-20°C). In one section, fibres were classified as Type I (light staining, slow myosin heavy chain) or Type II (dark staining, fast myosin heavy chain) based on their staining intensity for myosin ATPase after an alkaline (pH=9.4) pre-incubation (Nwyoye et al, 1982). Quantitative histochemical methods were used on subsequent serial sections to determine succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (GPD) enzyme activity according to the methods of Blanco et al. (1988) and Martin et al. (1985), respectively. Briefly, the SDH and GPD reactions were performed in 10.0 ml coplin jars as follows: SDH, 100 mM phosphate buffer, 10 μM sodium azide, 20 μM 1-methoxyphenazine methylsulphate, 1.5 mM nitro blue tetrazolium, 5mM EDTA and 48mM succinic acid, pH 7.6 for 8 minutes at room temperature; GPD, 100 mM phosphate buffer, 10 μM sodium azide, 20 μM 1-methoxyphenazine methylsulphate, 1.2

mM nitro blue tetrazolium, and 9.3 mM glycerophosphate, pH 7.4. in a water bath maintained at 37°C for 11 minutes. The sections were dried in the dark for 30 minutes before mounting with aquamount. Fibre cross-sectional area was determined from the fibres stained for myosin ATPase. Muscle fibres were selected from a centrally located region within the cross-section of the soleus and plantaris. The tibialis anterior was divided into a deep (mixed type I and II) and superficial (exclusively type II) portions using connective tissue as a landmark. A sample of 40 - 50 fibres was taken from the soleus, plantaris and from each region of the tibialis anterior for analysis. The same 40 - 50 fibres were identified and analyzed for ATPase, SDH and GPD activity from serial sections of each muscle. A cutoff filter was used to eliminate all light with a wavelength less than 450 nm in each density measurement.

Statistics: Analysis of variance (ANOVA) and Newman Keuls post-hoc comparison was used to test for significant differences in body weight, tibial length, muscle wet weight, fibre type composition, metabolite concentration and the ATP:ADP ratio at $p < 0.05$. The single fibre measurements of cross-sectional area, SDH and GPD were used to generate type I and II fibre mean values for each muscle. These values were then averaged over eight animals to determine the response of each treatment group. Separate 2x2 factorial ANOVAs were used to analyse the cross-sectional area, SDH and GPD data in the soleus and plantaris. A separate 2x3 factorial ANOVA was performed on the tibialis anterior which included type I and II fibres from the deep region and type II fibres from the superficial region. When significant interaction of main effects occurred a one-way ANOVA was used accompanied with Newman-Keuls post-hoc comparison of means to test for significant differences between individual means ($p < 0.05$).

2.3 Results

There were no significant differences in mean body weight between the C (247 ± 5 g) and GPA (241 ± 10 g) rats at the end of the 8 week pair-feeding program. Mean tibial length, a general index of growth (Yin et al., 1992), was also not different between the C (37.02 ± 0.22 mm) and GPA (36.95 ± 0.30 mm) rats.

Phosphocreatine and Adenine Nucleotides GPA feeding significantly decreased PCr and ATP concentration in the soleus, plantaris and the tibialis anterior (Table II-1). In contrast, the concentration of ADP was not significantly altered. This resulted in a significantly decreased ATP:ADP ratio in the soleus (C, 4.35 ± 0.08; GPA, 2.69 ± 0.14), the plantaris (C, 6.68 ± 0.46; GPA, 3.45 ± 0.36) and the tibialis anterior (C, 6.88 ± 0.22; GPA, 4.03 ± 0.22). In addition, GTP concentration was significantly decreased in the soleus, but not in the plantaris or tibialis anterior (Table II-1). The resting concentration of IMP was significantly decreased in the plantaris, but not in the soleus or tibialis anterior (Table II-1). The resting levels of AMP were detectable only as trace amounts in control muscles by the HPLC method used. In contrast, some values were obtained for GPA muscles; plantaris, 0.101 μmol/mg (n=2) and the tibialis anterior, 0.068 μmol/mg (n=3).

Muscle mass and fibre morphology

The mean wet weights of the soleus, plantaris and the tibialis anterior were significantly reduced ($p < 0.05$) 14 - 18% following GPA feeding (Table II-2). In the soleus and plantaris GPA feeding had no significant effect on fibre cross-sectional area (Table II-3). In contrast, fibre area was significantly reduced in the tibialis anterior following GPA feeding, but only in the type II fibres of the deep and superficial region (Figure II-1). The relative percentage of fibres classified as type I (light ATPase staining intensity) was increased significantly in the soleus and in the deep region of the tibialis anterior (Table II-2, Figures II-2, 4). The fibre type composition of the plantaris and of the superficial region of tibialis anterior were unchanged (Table II-2, Figure II-3).

Muscle fibre SDH and GPD activities

Single fibre measurements of GPD and SDH enzyme activities in representative muscles of C and GPA animals are presented in Figures II-5, II-6 and II-7. The range in enzyme activities and the distinctiveness in metabolic character between type I and II fibres is apparent from these Figures and in Table II-3 where in every case there is a significant fibre type difference in enzyme activity. From these single fibre measurements the mean enzymatic activities were determined for type I and II fibres from C and GPA rats (Table II-3). The GPD activity was increased following GPA feeding in the tibialis anterior, but not in the soleus or plantaris (Table II-3). In contrast, chronic ingestion of GPA increased the SDH activity in the soleus, plantaris and tibialis anterior. There were no fibre type specific differences in the adaptation of GPD or SDH activity to GPA feeding.

2.4 Discussion

It has been suggested that the morphological and metabolic adaptations associated with chronic feeding of the creatine analogue ß-guanidinopropionic acid are related to the muscle's predominant fibre type (Shoubridge et al., 1985; Petrofsky and Fitch, 1980) and/or mitochondrial density (Lai and Booth, 1990). To date these observations have been made on whole muscles or portions of muscles that are predominantly composed of one fibre type. From such observations, it is difficult to identify what factor(s) determine a muscle's adaptation to GPA feeding without insight into the changes occurring at the single fibre level. Therefore, this study was undertaken to describe the properties of single fibres in muscles that differed in functional, metabolic and contractile properties following GPA feeding.

In support of previous reports (Fitch et al. 1974, 1975), we observed that PCr, ATP and the ATP/ADP ratio were all decreased in whole muscle homogenates of the soleus, plantaris and tibialis anterior muscles of the GPA rats. A potential limitation of this study is that estimates of single fibre adenine nucleotide levels were not made in conjunction with single fibre enzyme measurements. However, Hucul et al. (1985) suggested that the absolute levels of nucleotides may be less important than the relative ratio of respective nucleotides within a muscle. Coupling this point with the essentially equivalent decrease in ATP across the soleus, plantaris and tibialis anterior muscles, it is reasonable to assume that whole muscle values generally reflect those of the single fibres.

In corroboration of earlier studies (Petrofsky and Fitch, 1975; Shoubridge et al., 1985; Lai and Booth, 1991) muscle wet weights were reduced following GPA feeding. However, we observed a 17% decrease in the plantaris wet weight, compared to a 30% decline reported by others (Petrofsky and Fitch, 1975; Shoubridge et al., 1985). Furthermore, we observed a decrease in soleus wet weight, whereas others have reported no change (Petrofsky and Fitch, Lai and Booth, 1991). There was no general growth suppression in our GPA rats since tibial length was unchanged. The reason for these discrepancies is not readily apparent. In GPA-fed animals, muscles with a high relative proportion of type IIb fibres (eg. "superficial" gastrocnemius) undergo the greatest changes in muscle mass and fibre cross-sectional area (Shoubridge et al. 1985; Shields et al. 1975). The data obtained from the tibialis anterior in the present study supports this observation. In the present study the decrease in muscle wet weight associated with GPA feeding cannot entirely be accounted for by a decrease in fibre size. However, GPA has not been known to alter the wet to dry ratio of muscle (Fitch et al., 1974).

The most consistent effect observed following GPA feeding was the increase in SDH activity in both fibre types in each of the muscles studied. However, this finding differs from those of Shoubridge et al. (1985) who reported no change in citrate synthase or oxoglutarate dehydrogenase activity in soleus whole muscle homogenates after 6-10 weeks of GPA feeding. In addition, cytochrome oxidase and citrate synthase activity in soleus muscles of mice fed GPA did not change relative to control (Moerland and Kushmerick, 1994). In support of the present data, Lai and Booth (1990) reported a significant increase in cytochrome c mRNA in the soleus after 3 and 11 weeks of GPA feeding. Our observed increase in SDH activity in the plantaris is consistent with a previous report of an increase in whole muscle homogenate citrate synthase and oxoglutarate dehydrogenase activity following GPA feeding

(Shoubridge et al., 1985).

The tibialis anterior was the only muscle to demonstrate a significant increase in GPD activity following GPA feeding. This observation taken in conjunction with the previously discussed morphometric and SDH activity data, suggest that relative to the soleus and plantaris, the tibialis anterior was most responsive to the GPA diet. Furthermore, it was apparent that muscles which vary in physiological and functional characteristics display different adaptations to GPA feeding when analyzed at the single fibre level. In a recent review, Roy et al. (1991) emphasized the disparate response of muscles and fibre types to a variety of perturbations. In each experimental model studied a functional rationale for the adaptive response of a muscle could be formulated based on differences in recruitment patterns or muscle loading characteristics (Roy et al., 1991). In the case of GPA feeding, intracellular energetics are pharmacologically altered, apparently independent of any change in neuromuscular activity or loading patterns (Moerland et al., 1989). Despite this fact, adaptations consistent with altered neuromuscular activity and/or loading have been observed (Shoubridge et al., 1980; Petrofsky and Fitch, 1975); present data). Therefore, it could be hypothesized that muscle fibre adaptations to an altered activity/load history may reflect a chronic change in cellular energetics. Recently, Green et al. (1992) also proposed that a depressed adenylate phosphorylation potential may be related to the adaptation of skeletal muscle to chronic electrical stimulation. Currently we are testing this hypothesis by investigating the effect of combinations of altered neuromuscular function and GPA feeding on muscle fibre properties and protein turnover (Marsh et al. 1992; Marsh and Martin 1993).

In summary, the results obtained indicate that muscle and fibre type specific adaptation occurs when PCr and ATP concentrations are depleted to a similar extent following GPA feeding. The percentage of fibres classified as type I increased in the soleus and deep portion of the tibialis anterior, but not in the plantaris. The cross-sectional area of fibres classified as type II in the plantaris and tibialis anterior was more susceptible to atrophy than type I fibres. In contrast to morphological adaptations, the succinate dehydrogenase activity was increased in each muscle in both type I and II fibres.

TABLE II-1. Phosphocreatine, adenine and guanidine nucleotides in the soleus, plantaris and tibialis anterior.

		CONTROL	GPA
SOL	PCr	11.09 ± 0.38	1.12 ± 0.35[a]
	ATP	3.56 ± 0.29	2.46 ± 0.33[a]
	ADP	0.82 ± 0.07	0.92 ± 0.13
	GTP	0.11 ± 0.01	0.06 ± 0.02[a]
	IMP	0.18 ± 0.03	0.18 ± 0.04
PL	PCr	13.94 ± 0.92	0.55 ± 0.09[a]
	ATP	5.82 ± 0.72	3.12 ± 0.40[a]
	ADP	0.87 ± 0.10	0.91 ± 0.09
	GTP	0.10 ± 0.02	0.07 ± 0.01
	IMP	0.38 ± 0.05	0.20 ± 0.03[a]
TA	PCr	15.33 ± 0.67	1.04 ± 0.08[a]
	ATP	7.06 ± 0.43	4.43 ± 0.22[a]
	ADP	1.03 ± 0.05	1.11 ± 0.09
	GTP	0.12 ± 0.01	0.10 ± 0.02
	IMP	0.34 ± 0.04	0.36 ± 0.05

Mean μmol/mg wet weight ± standard error of the mean (SEM), n=8. Abbreviations: GPA, ß-guanidinopropionic acid; SOL, soleus; PL, plantaris; TA, tibialis anterior; PCr, phosphocreatine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GTP, guanidine triphosphate; IMP, inosine monophosphate. [a], significantly different from control, $p < 0.05$.

TABLE II-2. Muscle wet weight and fibre morphology.

		CONTROL	GPA
SOL	WET WEIGHT (mg)	146 ± 4	120 ± 5[a]
	TYPE I (%)	77 ± 2	87 ± 2[a]
PL	WET WEIGHT (mg)	276 ± 8	230 ± 6[a]
	TYPE I (%)	18 ± 2	20 ± 2
TA	WET WEIGHT (mg)	510 ± 20	436 ± 14[a]
	TYPE I_{DEEP} (%)	5 ± 1	12 ± 2[a]

Mean ± SEM, n=10. Abbreviations are as in Table 1; Type I, fibres classified as type I. [a], significantly different from control, $p < 0.05$.

TABLE II-3. Single fibre area and enzyme activities of the soleus, plantaris and tibialis anterior.

SOLEUS

COMPARISON		CSA (μm^2)	GPD (OD/min)	SDH (OD/min)
GPA:	C	1607 ± 53	3.1 ± 0.4	28.0 ± 1.6
	GPA	1486 ± 53	2.3 ± 0.4	36.8 ± 1.6^{a}
FIBRE TYPE:	I	2015 ± 53	2.0 ± 0.4	27.5 ± 1.6
	II	1078 ± 53^{a}	3.4 ± 0.4^{a}	37.3 ± 1.6^{a}

PLANTARIS

COMPARISON		CSA (μm^2)	GPD (OD/min)	SDH (OD/min)
GPA:	C	1751 ± 66	6.8 ± 0.8	35.6 ± 1.7
	GPA	1572 ± 66	8.9 ± 0.8	45.1 ± 1.7^{a}
FIBRE TYPE:	I	1914 ± 66	5.9 ± 0.8	37.9 ± 1.7
	II	1408 ± 66^{a}	9.7 ± 0.8^{a}	42.7 ± 1.7^{a}

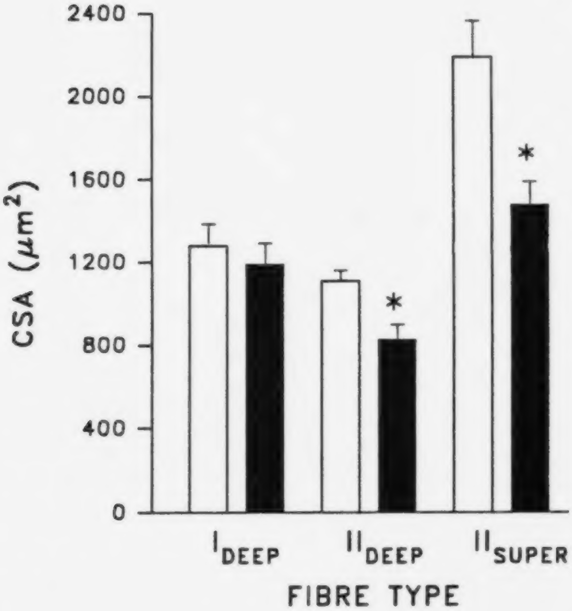
TIBIALIS ANTERIOR

COMPARISON		CSA (μm^2)	GPD (OD/min)	SDH (OD/min)
GPA:	C	1529 ± 59	7.4 ± 5.5	30.1 ± 1.8
	GPA	1165 ± 59^{a}	9.2 ± 5.5^{a}	44.5 ± 1.8^{a}
FIBRE TYPE:	I	1236 ± 69	5.1 ± 6.5	39.3 ± 2.1
	II_{DEEP}	968 ± 69	9.2 ± 6.5	48.2 ± 2.1
	II_{SUPER}	1838 ± 78^{ab}	10.6 ± 7.3^{a}	24.4 ± 2.4^{a}

Mean ± SEM, n=20. Abbreviations: as in Table II-1; CSA, cross-sectional area; GPD, α-glycerophosphate dehydrogenase; SDH, succinate dehydrogenase. ^{a}, significant main effect; ^{b}, significant interaction of main effects; $p < 0.05$.

FIGURE II–1. Cross–sectional area of type I and type II fibres of control and GPA–fed tibialis anterior. *, significantly different . from control.

☐ control rats. ■ GPA–fed rats.



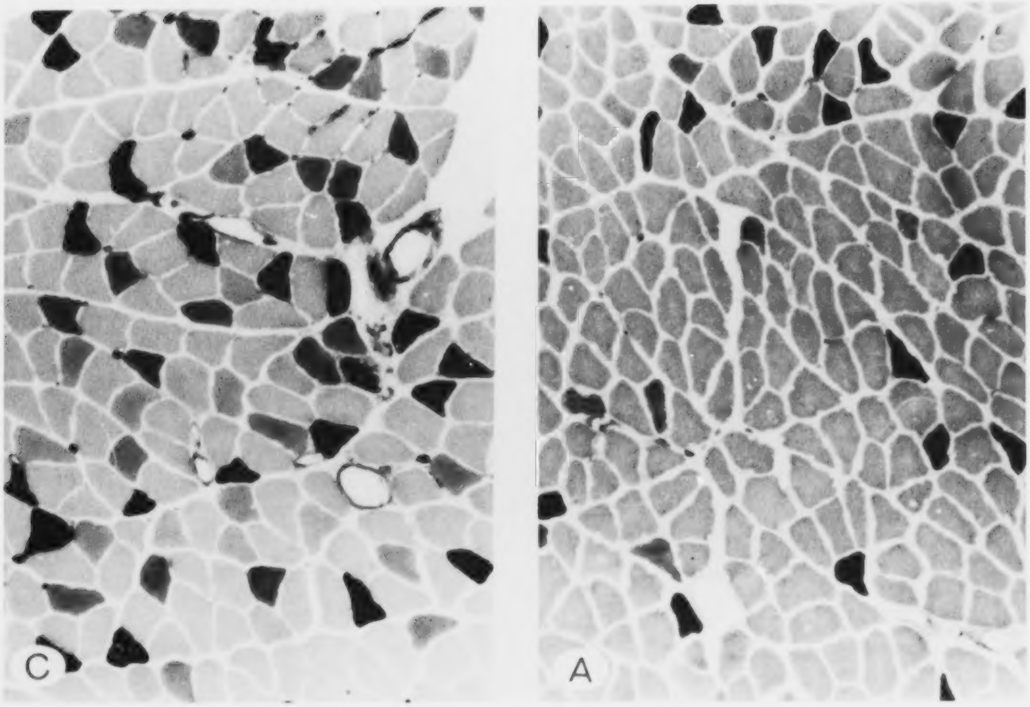


Figure II-2. Myofibrillar ATPase staining reaction of control (C) and GPA-fed (A) soleus. Type II fibres are darkly stained. See text for methodological details. Magnification of tissue was 10x with subsequent print enlargement of 3x.

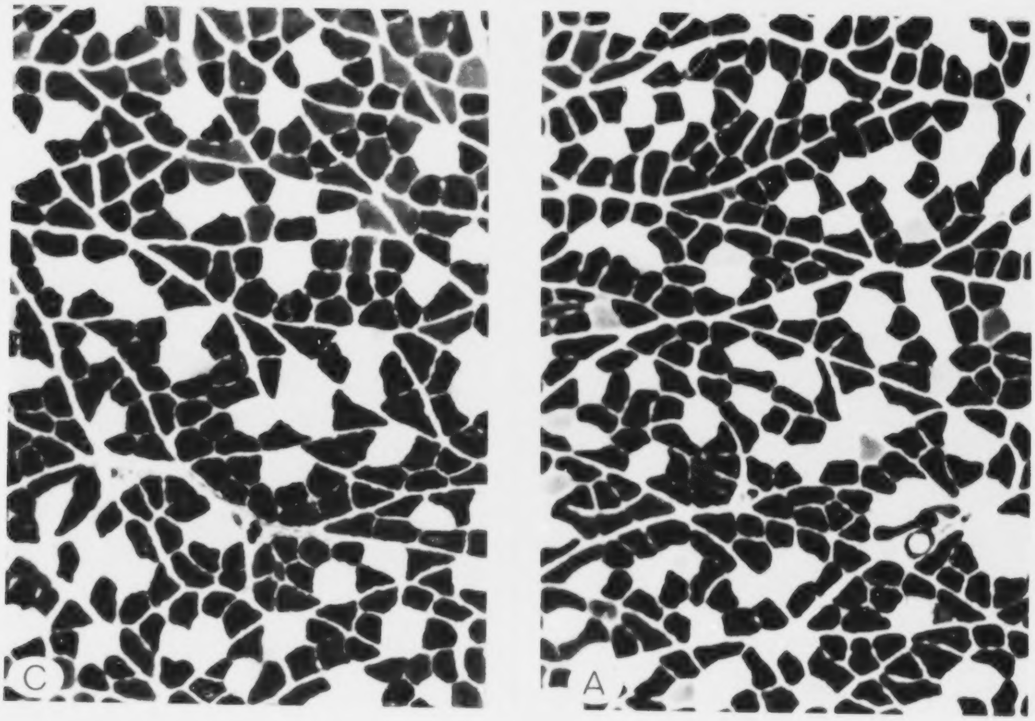


Figure II-3. Myofibrillar ATPase staining reaction of control (C) and GPA-fed (A) plantaris. Type II fibres are darkly stained. See text for methodological details. Magnification of tissue was 10x with subsequent print enlargement of 3x.

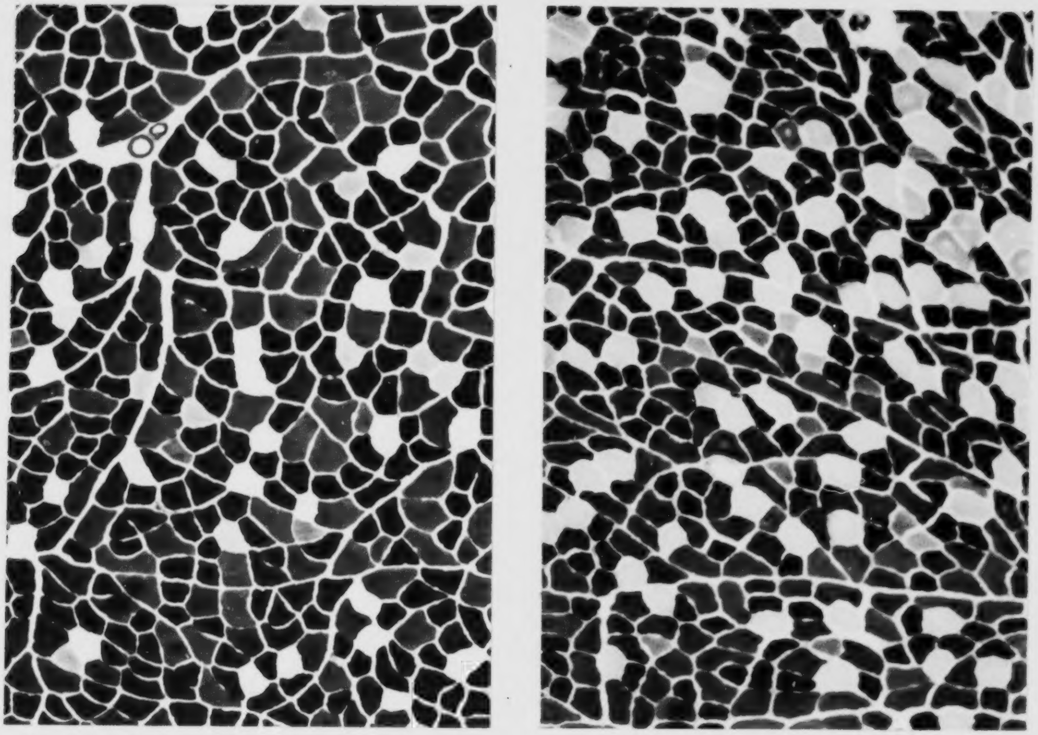


Figure II-4. Myofibrillar ATPase staining reaction of the deep region of the tibialis anterior. Control muscle is on the left and GPA-fed on the right side of page. Type II fibres are darkly stained. See text for methodological details. Tissue was photographed at magnification 10x with subsequent print enlargement of 3x.

FIGURE II–5. Single fibre measurements of enzyme activity in representative soleus of control and GPA–fed rats.
○ Type I fibres; ● Type II fibres.

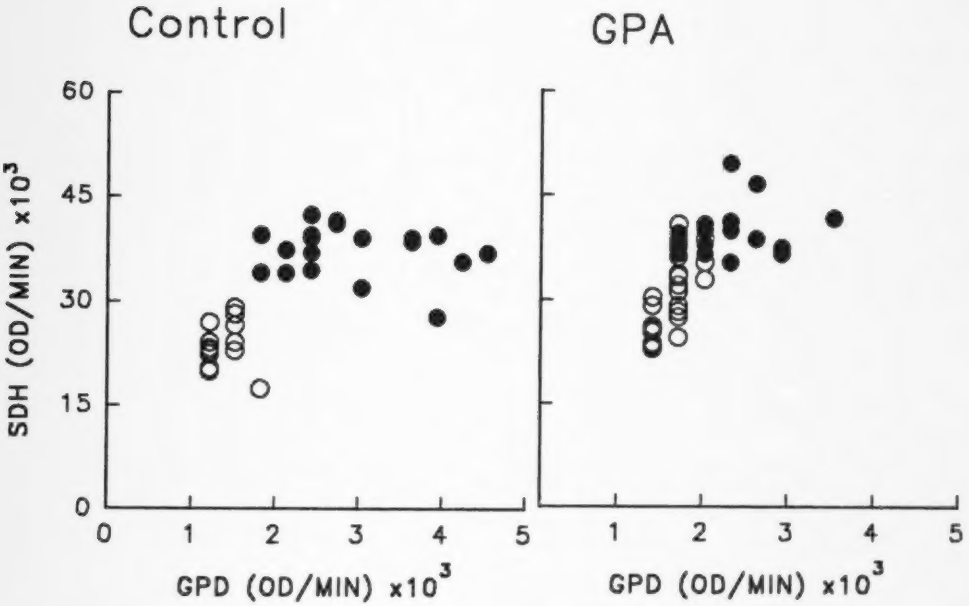


FIGURE II–6. Single fibre measurements of enzyme activity in representative plantaris of control and GPA–fed rats.
○ Type I fibres; ● Type II fibres.

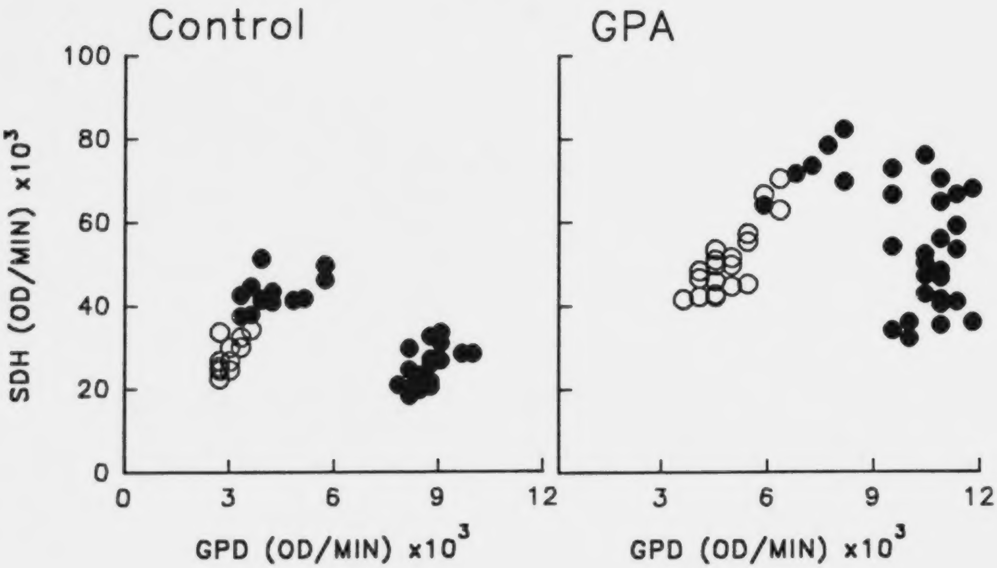
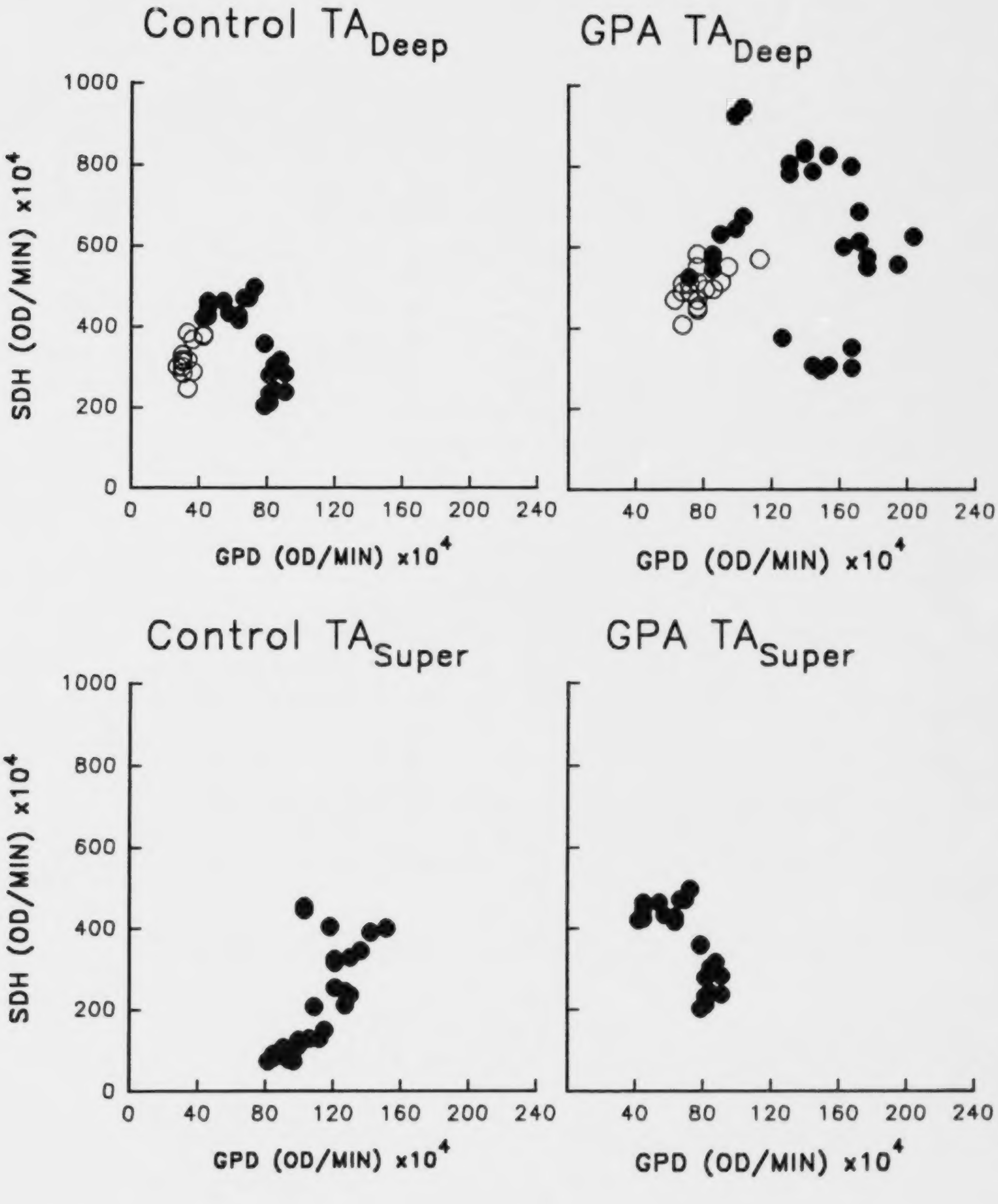


FIGURE II–7. Single fibre measurements of enzyme activity in representative tibialis anterior of control and GPA–fed rats.
○ Type I fibres; ● Type II fibres



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CHAPTER III

Adaptation to synergist ablation in the rat plantaris depleted of high energy phosphates.[2]

3.1 Introduction

When the gastrocnemius and soleus muscles of the triceps surae muscle group are surgically removed (synergist ablation), the recruitment level and subsequent EMG activity of the plantaris is increased without altering its temporal pattern of activation (Gardiner et al., 1986; Roy et al., 1991). In the rat, the increased functional demand from normal weight-bearing activities is sufficient to stimulate hypertrophy of type I and II muscle fibres. In addition, the metabolic and contractile properties of the hypertrophied plantaris suggest a shift towards that of a slow-twitch muscle (Ianuzzo and Chen, 1979; Roy et al., 1982, 1991; Baldwin et al., 1982). It has been postulated that an increase in the activation and subsequent development of muscle fibre tension is necessary to stimulate fibre hypertrophy (Roy et al., 1991). In contrast, mitochondrial enzyme activities are thought to be more sensitive to the activity patterns and associated metabolic demands of the muscle fibre (Pette and Vrbova, 1985; Roy et al., 1991). Thus, the stimulus for muscle hypertrophy and the stimulus for increased mitochondrial enzyme activity may be incompatible. The so called "interference effect" is said to occur when resistance strength training and endurance training are combined and produce compromised results.

It is possible that a common mechanism exists by which the morphological and metabolic properties of skeletal muscle are regulated. During muscle activity and tension development hydrolysis of ATP and PCr occurs. When muscle contraction intensity is low, creatine kinase activity and mitochondrial ATP synthesis resist a decrease in ATP concentration. However, when contraction intensity increases, creatine kinase activity and mitochondrial ATP synthesis cannot match ATP demand and ATP concentration decreases. The products of ATP hydrolysis and the adenylate phosphorylation potential ($ATP/ADP \cdot P_i$) are known to be important regulators of skeletal muscle metabolism at rest and during exercise (Newsholme and Leach, 1983). In addition, chronic decreases in the adenylate phosphorylation potential have been postulated to regulate myocardial adaptation to increased functional demand by stimulating mitochondrial biogenesis and synthesis of structural proteins (Meerson, 1975). Furthermore, a chronic depression of the adenylate phosphorylation potential has recently been proposed to be an important signal for muscle fibre transformation during chronic electrical stimulation (Pette and Düsteroft, 1992; Green et al., 1992). It is unknown whether the adenylate system may play a causative role or merely be a secondary response during adaptation to increased functional demand.

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[2] Co-authors include T. P. Martin - discussion and editing of manuscript.

When rats are fed a diet containing 1-2% of the creatine analogue ß-guanidinopropionic acid (GPA) the concentration of phosphocreatine (PCr) and adenosine triphosphate (ATP) are reduced by 90% and 50% respectively (Fitch et al., 1974, 1975). In contrast, the resting levels of ADP and inorganic phosphate (P_i) were not altered by GPA feeding in the tibialis anterior (Fitch et al., 1975). Thus, GPA feeding results in a chronic decrease in adenylate phosphorylation potential. The purpose of this study was to use GPA feeding as an experimental tool to investigate the importance of high energy phosphates during adaptation of type I and II muscle fibres to an increased functional demand. It was hypothesized that a reduction in PCr and ATP would attenuate the morphological and metabolic adaptations to synergist ablation in muscle fibres of the rat plantaris classified as type I or type II.

3.2 Methods

Animals: Female Sprague-Dawley rats (mean 200 g) were singly housed in a light and temperature controlled environment (12h light/dark, 24°C) in accordance with the guidelines of the Canadian Council on Animal Care. Rats were weight-matched prior to pair feeding and randomly assigned between four groups; control (C), synergist ablated (SA), 2% GPA diet (GPA) and 2% GPA diet combined with synergist ablation (GPA/SA). Pair feeding was based on the weight of food consumed per day by the GPA animals. GPA was synthesized by the method of Rowley et al. (1971) and the crystals were then powdered and mixed dry with ground rat chow.

Compensatory Hypertrophy: After four weeks of feeding either the control, or GPA diet, SA and GPA/SA rats were anaesthetized by inhalation of Metafane and the soleus and gastrocnemius muscles were bilaterally removed. Each animal received an intramuscular injection of an antibiotic (Derapen-C) and an analgesic (Buprenorphin) and maintained on their respective diets for an additional 4 weeks. Thus, the duration of muscle adaptation to synergist ablation was 4 weeks.

Tissue Preparation: Following eight weeks of feeding and four weeks of synergist ablation, the rats from each group were weighed and anaesthetized with sodium pentobarbitol (35 mg/kg). The plantaris muscles were removed from each limb and immediately frozen in liquid nitrogen-chilled isopentane and stored at -70°C. Each frozen muscle was weighed, the mid-belly sectioned, placed in OCT embedding medium and frozen onto cork in liquid nitrogen. This portion of muscle was used for quantitative histochemical analyses. The distal and proximal ends of the muscle were powdered under liquid nitrogen and freeze-dried. All other muscles were removed from the limb and the length of each tibia was then measured with a micrometer.

Phosphocreatine and adenosine triphosphate estimation: Following an extraction of the freeze-dried tissue (0.5 M $HClO_4$, 1mM EDTA) and neutralization (2.2 M $KHCO_3$), the concentration of ATP and PCr were determined spectrophotometrically (Harris et al., 1974).

Histochemical Analyses: Each plantaris was cut into 10 μm thick serial sections with a cryostat (-20°C). In one section, muscle fibres were classified as

Type I (light staining) or Type II (dark staining) based on their staining intensity for myosin ATPase, alkaline pre-incubation, pH=9.4 (Nwyoye et al., 1982). Quantitative histochemical methods were used on subsequent serial sections to determine succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (GPD) enzyme activity according to Blanco et al. (1988) and Martin et al. (1985), respectively. Briefly, the SDH and GPD reactions were performed in 10.0 ml coplin jars as follows: SDH, 100 mM phosphate buffer, 10 μM sodium azide, 20 μM 1-methoxyphenazine methylsulphate, 1.5 mM nitro blue tetrazolium, 5 mM EDTA and 48 mM succinic acid, pH 7.6 for 8 minutes at room temperature; GPD, 100 mM phosphate buffer, 10 μM sodium azide, 20 μM 1-methoxyphenazine methylsulphate, 1.2 mM nitro blue tetrazolium, and 9.3 mM glycerophosphate, pH 7.4, in a water bath maintained at 37°C for 11 minutes. The sections were dried in the dark for 30 minutes before mounting with aquamount. Fibre cross-sectional area was determined from the fibres stained for myosin ATPase. The same 40 - 50 fibres were identified and analyzed from each serial section to generate type I and II fibre mean GPD, SDH and cross-sectional area for each muscle. To avoid region specific loading of muscle fibres, care was taken to examine centrally located type I and II fibres; not peripheral type II and deep type I fibres. The product of fibre cross-sectional area and enzyme activity (Chalmers et al., 1992) was calculated to estimate the total enzymatic capacity of each fibre.

Statistics: Multifactorial analysis of variance (2x2) was used to analyze the data and test for significant main effects of GPA feeding and synergist ablation as well as significant interaction of main effects. The single fibre measurements of cross-sectional area, SDH and GPD were used to generate type I and II fibre mean values for each muscle. Separate 2x2 factorial ANOVAs were used to analyze the data from type I and type II fibres. Significance was accepted for main effects and interaction with a 0.95 confidence level. When significant interaction was present, a one-way ANOVA and subsequent Newman-Keuls test were used to compare individual group means at $p < 0.05$.

3.3 Results

There were no significant differences in body weight between the groups at the end of the 8 wk feeding program. Mean tibial length, a general index of growth, was also not different between the groups. As expected, GPA feeding significantly decreased ATP and PCr concentration by 46% and 93%, respectively (Table III-1). Synergist ablation had no effect on the concentration of ATP or PCr.

Muscle mass and fibre morphology: Muscle wet weight was not altered by GPA feeding. In contrast, synergist ablation increased plantaris wet weight by 133% (684 g from 276 g) in compensatory hypertrophy (SA) rats and by 155% (586 g from 230 g) in GPA/SA rats. This whole muscle hypertrophy was also evident at the single fibre level as the cross-sectional area of type I and II fibres was significantly increased following synergist ablation (Table III-2). There was no significant interaction of GPA feeding and synergist ablation on cross-sectional area. The percentage of fibres classified as type I was not altered following GPA feeding or synergist ablation (Table III-1).

GPD and SDH activities: Figure III-1 illustrates the range in quantitative histochemical measurements of single fibre SDH and GPD enzyme activities in representative muscles of C, GPA, SA and GPA/SA animals. The GPD enzyme activity of plantaris muscle fibres was not altered following GPA feeding, whereas, synergist ablation significantly reduced GPD activity in both type I and II fibres (Table III-2). GPA feeding and synergist ablation did not affect the SDH activity of type I and II fibres similarly. In type I fibres, GPA feeding had no effect on SDH activity, synergist ablation significantly reduced SDH activity and there was no interaction of these main effects. The reduction in SDH activity following synergist ablation in type I fibres was due to a 10% and 26% decrease in SA and GPA/SA fibre SDH activity, respectively. In contrast, SDH activity of type II fibres was significantly increased by GPA feeding, whereas synergist ablation decreased SDH activity, but only when combined with GPA feeding (Table III-2, Figure III-2). The SDH activity of GPA/SA rat type II fibres was reduced by 33% compared to no significant change in the type II fibre SDH activity of SA rats. The total SDH activity per fibre was significantly increased in type I and II fibres following synergist ablation (Table III-2). Interestingly, this differs from total fibre GPD which remained similar to control values following synergist ablation (Table III-2). Total SDH activity of type II fibres increased following synergist ablation to a greater extent in control than GPA rats (Figure III-3). Total SDH activity per fibre was increased by 58% and 84% in type I and II fibres, respectively, of SA rats. In comparison, total SDH activity was not significantly increased in type II fibres and only increased by 25% in type I fibres of GPA/SA rats.

3.4 Discussion

The two main elements of this study, GPA feeding and synergist ablation, produced their desired effects; namely depletion of high energy phosphates and muscle fibre hypertrophy. The extent to which ATP and PCr concentration was decreased following GPA feeding was similar to previous reports (Fitch et al., 1974, 1975). The similar degree of hypertrophy in type I and II fibres (109% and 107%, respectively) observed following synergist ablation in this study differs from the results of Olha et al. (1988) who reported 109% hypertrophy in type I fibres versus only a 67% increase in type II fibre area. Olha et al. (1988) waited 12 - 16 weeks following surgical removal of the synergists prior to data collection compared to only 4 weeks in this study. However, Michel et al., (1989) reported a 103% increase in type I fibre area, but only a 20% increase in type II fibre area 4 weeks after synergist ablation. The reason for the variance in relative hypertrophy of type II fibres to type I fibres is unclear, but may be attributable to the sampling region of type II fibres. Regional differences in hypertrophy have been reported (Roy et al., 1991) and have been attributed to differential recruitment of muscle sub-regions. In the present study, type I and II fibres were both sampled from the same central region of the muscle cross-section.

The objective of this study was to determine the importance of normal resting levels of ATP and PCr during compensatory hypertrophy of skeletal muscle following synergist ablation. Despite the fact that GPA feeding and synergist ablation each produced their desired effects, compensatory hypertrophy was not attenuated when synergist ablation was combined with GPA feeding. In addition, the GPD activity of type I and II fibres was reduced by 40% following synergist ablation in both C and GPA-fed muscle which is similar to previously reported values (Baldwin et al., 1982). In corroboration of a previous report (Ianuzzo and Chen, 1979), SDH activity was unchanged in type I and II fibres following synergist ablation. However, the combination of synergist ablation and GPA feeding decreased SDH activity. Furthermore, when synergist ablation was combined with GPA feeding, total SDH activity failed to increase to the same extent as control.

The enzyme SDH is bound to the inner mitochondrial membrane (Newsholme and Leach, 1984). Therefore, the histochemical determination of SDH activity can also serve as an indicator of mitochondrial volume (Hood et al., 1990). The observation that SDH activity following synergist ablation remains constant relative to control (Figure III-2) is evidence that SDH activity, and perhaps mitochondrial volume as well, increases in concert with fibre volume. However, in type II fibres of GPA/SA rats this is not the case. There is actually a decrease in SDH activity following synergist ablation of GPA-fed plantaris. Thus, it appears that the depletion of ATP and PCr subsequent to GPA feeding combined with synergist ablation lowers SDH enzyme activity and perhaps limits mitochondrial biogenesis. Total SDH activity per fibre (Figure III-3) also supports this premise. Although total SDH activity per fibre does increase in GPA/SA plantaris, it fails to increase to the same extent as synergist ablation of control muscle. In contrast to total SDH activity, total GPD activity does not change following synergist ablation. Therefore, the expression of this glycolytic enzyme is not coordinated with fibre volume, but instead its specific activity is "diluted" by the increase in fibre volume following synergist ablation.

The adenylate phosphorylation potential has been suggested to be an integral component in a putative adaptive mechanism which matches ATP synthesis to increased functional demand (Meerson, 1975). Meerson (1975) has proposed that chronic depression of the ATP phosphorylation potential stimulates mitochondrial biogenesis to resist further fluctuations in ATP concentration. Previous reports are conflicted as to whether muscle contraction affects the concentration of ATP and the subsequent adenylate phosphorylation potential in a similar fashion in GPA-fed muscle compared to control muscle (Fitch et al., 1975; Meyer et al., 1986; Shoubridge and Radda, 1987). It is possible that the adaptive signals generated subsequent to synergist ablation differed enough between GPA-fed muscle and control muscle to decrease the SDH enzyme and perhaps mitochondrial biogenesis. Furthermore, the decreased ATP and PCr concentration may have altered the response to synergist ablation by reducing contractile activity during the overload stimulus. An alternative explanation is that mitochondrial biogenesis is energy-dependent and the decrease in ATP and PCr were rate limiting for protein translation. However, it is beyond the scope of this chapter to address any of the complexities involved in transcriptional, translational or post-translational regulatory mechanisms of gene expression.

It has recently been demonstrated that contractile economy and aerobic recovery metabolism improves in muscle following GPA feeding (Moerland and Kushmerick, 1994). Following synergist ablation and the subsequent increase in EMG activity (Gardiner et al., 1986) contractile economy and improved aerobic metabolism would be practical adaptations for the hypertrophying plantaris. Moerland and Kushmerick (1994) attribute the increased contractile economy to fast to slow isomyosin transitions and the improved aerobic recovery to increased activity of mitochondrial enzymes. When synergist ablation is superimposed on GPA-fed muscle which already possesses increased contractile economy and mitochondrial enzyme activity, the muscle would appear to be predisposed to the increased contractile demands of functional overload. However, that is not the case. Instead of maintaining the GPA-induced elevated SDH activity, muscle adapts to synergist ablation by decreasing SDH activity and increasing type I and II fibre cross-sectional area. In conclusion, the stimuli for fibre hypertrophy and increasing mitochondrial enzyme activity appear to be dissimilar. Decreasing ATP and PCr concentrations increases mitochondrial enzyme activities and reduces fibre area whereas, synergist ablation increases fibre cross-sectional area and has no effect on mitochondrial enzyme activity. Adaptation to the combination of GPA feeding and synergist ablation appears to support the so called "interference effect" as mitochondrial enzyme activity was lost as fibre hypertrophy occurred.

TABLE III-1. Muscle morphology and high energy phosphate concentration.

Main effects	GPA		SA	
Comparison	C	GPA	C	SA
ATP	21.1 ± 0.8	12.1 ± 0.7[a]	17.0 ± 1.0	16.1 ± 0.6
PCr	55.3 ± 2.3	3.9 ± 0.6[a]	30.1 ± 1.5	29.1 ± 1.4
MASS	480 ± 21	408 ± 20	253 ± 19	635 ± 22[a]
Type I	18 ± 2	21 ± 2	19 ± 2	21 ± 2

Mean ± SEM. Main effects; GPA, rats fed ß-guanidinopropionic acid; SA, rats synergist ablated. Left column; Control and SA groups combined (C, n=14) vs. GPA-fed and GPA/SA groups combined (GPA, n=14). Right column; Control and GPA groups combined (C, n=16) vs. SA and GPA/SA groups combined (SA, n=12). Abbreviations: MASS, wet weight of plantaris (mg); Type I, percentage type I fibres; ATP, adenosine triphosphate (μmol/mg dry weight); PCr, phosphocreatine (μmol/mg dry weight); [a], significant main effect, $p < 0.05$.

Table III-2. Single fibre area and enzyme activities.

Main effects	GPA		SA	
	C	GPA	C	SA
Type I Fibres				
CSA	2971 ± 150	2584 ± 140	1914 ± 122	3641 ± 165[a]
GPD	3.9 ± 0.7	5.5 ± 0.7	5.9 ± 0.6	3.4 ± 0.8[a]
TG	10.1 ± 1.4	13.0 ± 1.3	11.2 ± 1.2	11.9 ± 1.6
SDH	33.0 ± 2.0	35.8 ± 2.1	37.9 ± 1.9	30.9 ± 2.3[a]
TS	84.7 ± 5.7	88.2 ± 5.9	72.0 ± 5.1	100.9 ± 6.4[a]
Type II Fibres				
CSA	2434 ± 104	1805 ± 97[a]	1408 ± 85	2830 ± 114[a]
GPD	6.8 ± 0.9	8.9 ± 0.8	9.7 ± 0.7	5.9 ± 1.0[a]
TG	15.1 ± 1.5	14.8 ± 1.4	13.5 ± 1.2	16.4 ± 1.7
SDH	35.4 ± 2.1	40.9 ± 2.2[a]	42.7 ± 1.9	33.6 ± 2.3[ab]
TS	82.1 ± 5.1	68.5 ± 5.4	59.1 ± 4.7	91.5 ± 5.8[ab]

Mean ± SEM. Main effects; GPA, rats fed β-guanidinopropionic acid; SA, rats synergist ablated. Left column; Control and SA groups combined (C, n=14) vs. GPA-fed and GPA/SA groups combined (GPA, n=14). Right column; Control and GPA groups combined (C, n=16) vs. SA and GPA/SA groups combined (SA, n=12). Abbreviations: CSA, cross-sectional area (μm^2); GPD, α-glycerophosphate dehydrogenase activity ((OD/min) x 10^3); SDH, succinate dehydrogenase activity ((OD/min) x 10^3); TG, and TS, total enzyme activity for GPD and SDH (OD/min x μm^2); [a], significant main effect; [b], significant interaction; $p < 0.05$.

FIGURE III−1. Single fibre measurements of enzyme activity in the normal and hypertrophied plantaris. ○ Type I fibres ● Type II fibres.

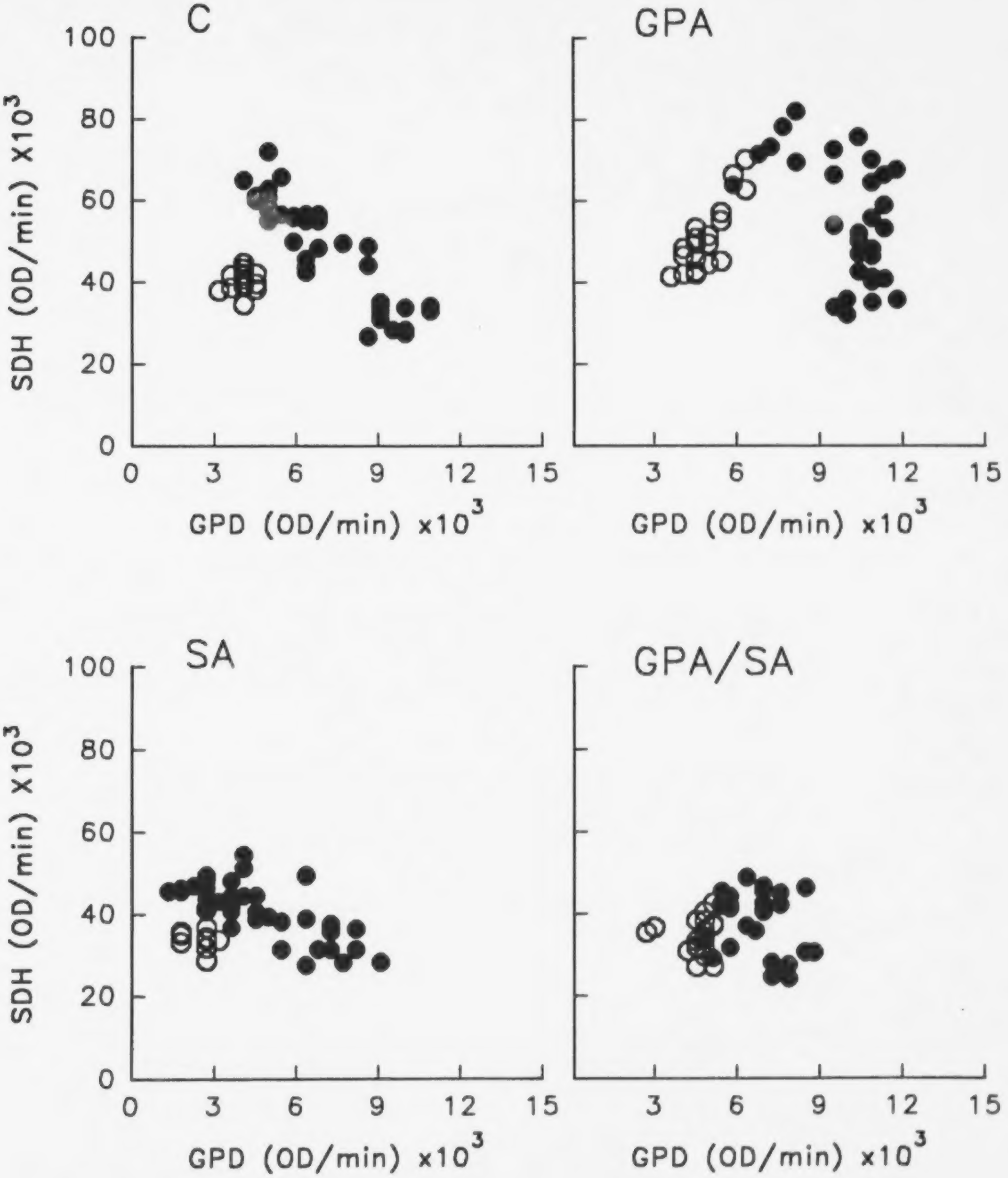


FIGURE III–2. SDH activity in type II fibres of control, GPA–fed, synergist ablated (SA) and GPA–fed combined with synergist ablation (GPA/SA) *, significantly different from control. □ control rats. ■ GPA–fed rats.

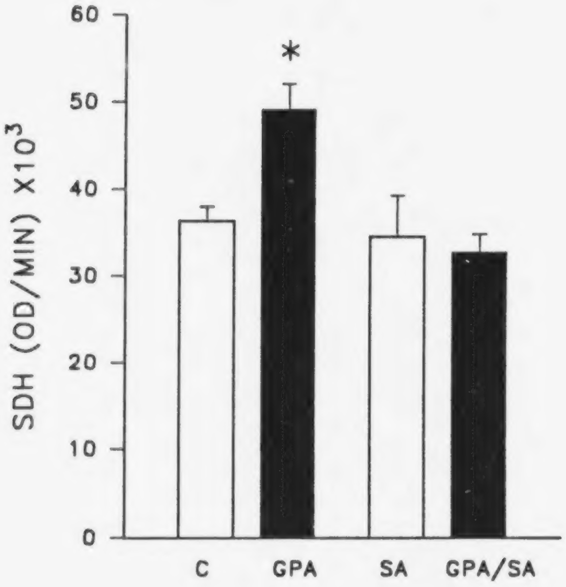
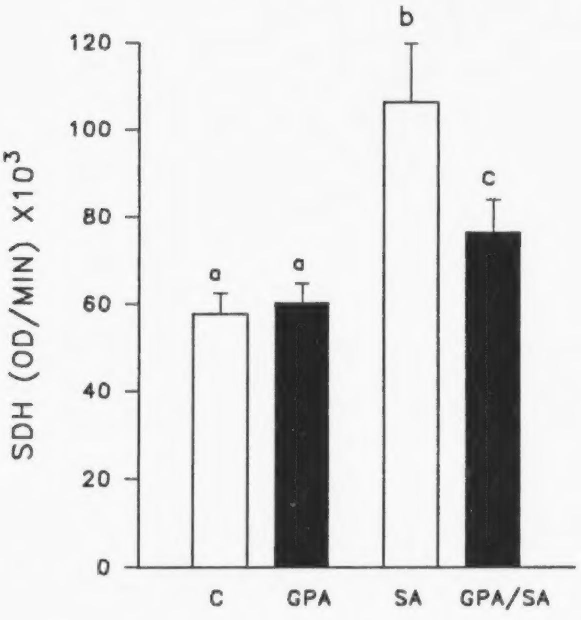


FIGURE III–3. Total SDH activity (product of SDH activity and CSA) in type II fibres of control, GPA–fed, synergist ablated (SA) and GPA–fed combined with synergist ablation (GPA/SA) rats. Bars with different letters are significantly different from eachother $p < 0.05$ ☐ control rats. ■ GPA–fed rats.



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CHAPTER IV

Adaptation of skeletal muscle to tetrodotoxin-induced paralysis in normal and ß-guanidinopropionic acid fed rats:

1. Effect on single fibre properties.[3]

4.1 Introduction

Neuromuscular activation and the subsequent development of tension in muscle is an important factor in maintaining the myofibrillar, sarcoplasmic and metabolic proteins characteristic of slow- and fast-twitch muscles (reviewed by; Pette and Vrbova, 1984; Roy et al., 1991). When neuromuscular activity is reduced, the response of slow- and fast-twitch muscles can vary. Slow-twitch postural muscles, such as the soleus, depend on chronic neuromuscular activity to maintain slow myosin heavy chain expression, low sarcoplasmic reticulum Ca^{2+}-ATPase activity and minor amounts of the Ca^{2+} binding protein parvalbumin (Gundersen et al., 1988; Leberer et al. 1986). In contrast, fast myosin heavy chain expression, high sarcoplasmic reticulum Ca^{2+}-ATPase activity and abundant parvalbumin in fast-twitch muscle is dependent on phasic neuromuscular activation (Gundersen et al., 1988; Leberer et al. 1986). When the low frequency, chronic activity pattern of a slow-twitch muscle is superimposed upon a fast-twitch muscle by electrical stimulation a phenotypic transformation occurs. An increase in slow myosin heavy chain, a decrease in sarcoplasmic reticulum Ca^{2+}-ATPase activity and a reduction in parvalbumin is observed such that the properties of the fast-twitch muscle resemble those of a slow-twitch muscle (Pette and Vrbova, 1984).

A similar phenotypic transition of fast-twitch muscle occurs when rats are fed a diet containing the creatine analogue ß-guanidinopropionic acid (GPA)

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[3] Co-authors include T. P. Martin and V. E. Baracos - discussion and editing of manuscript.

(Moerland et al., 1989). As in chronic electrical stimulation, GPA feeding has been shown to reduce levels of parvalbumin and induce isomyosin transitions (Moerland et al., 1989). In addition, the fibre type composition, contractile properties and metabolic enzyme activity have also been altered (Petrofsky and Fitch, 1980; Shoubridge et al., 1985). In general, skeletal muscle phenotype is transformed to favour slow-twitch, oxidative metabolism. Chronic ingestion of GPA reduces the intracellular concentration of phosphocreatine (PCr) and adenosine triphosphate (ATP) (Fitch et al., 1974, 1975) because GPA-P has a higher Km and a much lower Vmax as a substrate for creatine kinase than does PCr (Chevli and Fitch, 1979). The changes in the intracellular concentrations of PCr, ATP and ATP phosphorylation potential which occur during chronic ingestion of GPA have been postulated to play a role in mediating skeletal muscle adaptation (Lai and Booth, 1990; Moerland et al., 1989). In addition, a chronic depression of ATP phosphorylation potential has been associated with morphological and metabolic adaptations following chronic electrical stimulation (Green et al., 1992).

The morphological and metabolic adaptations which occur following GPA feeding may be independent of neuromuscular activation. In contrast to chronic electrical stimulation of muscle, the changes in muscle properties following GPA feeding may be solely attributable to the pharmacological reduction of the intracellular concentration of PCr and ATP. Moerland et al. (1989) report that isomyosin transitions and changes in parvalbumin content in murine muscle were not accompanied by any change in spontaneous activity or levels of thyroid hormone. Thus, it appears that chronic GPA may have a pharmacological effect on skeletal muscle properties that is independent of activity patterns and thyroid hormone levels. To test this hypothesis, ingestion of GPA was used in conjunction with a functional perturbation; neuromuscular paralysis induced by tetrodotoxin (TTX). TTX-induced neuromuscular paralysis decreases oxidative capacity, causes marked atrophy of fibre area and increases type II fibre composition in muscle (Spector, 1985; St.-Pierre et al., 1988; Gardiner et al., 1992). Therefore, following treatment of muscle with GPA, TTX or GPA + TTX, the activity of glycolytic and oxidative enzymes can be combined with measurements of muscle morphology and be used as indices of adaptation. Muscles that vary in functional, metabolic and contractile properties were examined because both GPA feeding and decreased neuromuscular activity may have fibre-type and/or muscle specific effects. Individual muscle fibres from the soleus (predominantly type I, ankle plantar flexor), plantaris (predominantly type II, ankle plantar flexor), and tibialis anterior (ankle dorsiflexor with a mixed type I and II deep region and a type II superficial region) were examined. Furthermore, the TTX model of neuromuscular inactivity has been used primarily to investigate the contractile and morphological properties of the soleus (Spector, 1985a, 1985b) and the gastrocnemius (St. Pierre and Gardiner, 1985; St. Pierre et al., 1988; Gardiner et al., 1992). The ankle dorsiflexors have not been examined in any detail and only a few analyses of the plantaris (Michel and Gardiner, 1990) have been undertaken. Therefore, the effects of TTX-induced paralysis on the morphological and metabolic properties of the soleus, plantaris, and tibialis anterior were examined. In addition, the extent of adaptation to TTX-induced paralysis was compared between control and GPA-fed muscle.

4.2 Methods

Animals: Female Sprague-Dawley rats were singly housed in a light and temperature controlled environment (12h light/ dark, 24°C and randomly assigned to four groups (n=8/group); control (C), tetrodotoxin (TTX)-induced paralysis (TTX), 2% GPA diet (GPA), and TTX paralysis combined with GPA feeding (GPA/TTX). The GPA was synthesized by the method of Rowley et al. (1971). The crystals were powdered and then mixed dry with ground rat chow and fed ad libitum to GPA and GPA/TTX rats for 8 weeks. Pair feeding was based on the weight of food consumed per day by the GPA animals. To determine if GPA feeding or TTX-induced paralysis suppressed growth, tibial length was measured as an index of growth (Yin et al. 1982).

TTX-Induced Paralysis: The neurotoxin TTX was chosen to reduce neuromuscular activity because TTX-induced paralysis does not disrupt axoplasmic transport or spontaneous release of acetylcholine. This results in significant differences in membrane potential, sarcoplasmic reticulum and metabolic properties of skeletal muscles following TTX-induced paralysis compared to denervation (Bray et al., 1979; Wan and Boegman, 1981a, 1981b). After four weeks of either the Control or GPA diet, TTX and GPA/TTX animals were anaesthetized by inhalation of Metafane and an osmotic pump (Alza Corp., model 2ML4), silastic catheter were implanted subcutaneously and the nerve cuff placed around the sciatic nerve (St. Pierre and Gardiner, 1985). The TTX (80 μg/ml saline) perfused the sciatic nerve at a constant rate (0.5 μl/hr) for four weeks. The contralateral limb was undisturbed. Each of the rats continued on their respective diets (control or GPA) for the remainder of the 8 week period. Before a paralysed muscle was included for analysis, the sciatic nerve was stimulated superior to the TTX cuff. If a contraction was elicited the muscles were excluded from analysis. The sciatic nerve was also stimulated distal to the cuff to ensure no denervation had occurred as a result of the cuff. If contraction was absent then the muscles were not included for analysis.

Tissue Preparation: Rats were anaesthetized with sodium pentobarbital (60 mg/kg) and the soleus, plantaris, and tibialis anterior muscles were bilaterally removed and immediately frozen in liquid nitrogen-chilled isopentane. The frozen muscles were weighed, a section of each muscle's mid-belly was removed, placed in OCT embedding medium and frozen onto cork in liquid nitrogen. This portion of muscle was used for quantitative histochemical analyses. The distal and proximal ends of the muscle were powdered under liquid nitrogen and freeze-dried. Connective tissue was separated from muscle tissue while being powdered under liquid nitrogen and after freeze-drying to reduce dilution of metabolite measurements. All other muscles were removed from the limb and the tibia was disarticulated and its length measured with a micrometer.

Phosphocreatine and adenosine triphosphate estimation: Following an extraction of the freeze-dried tissue (0.5 M $HClO_4$, 1mM EDTA) and neutralization (2.2 M $KHCO_3$) procedure, the concentration of ATP and PCr were determined spectrophotometrically (Harris et al. 1974).

Histochemical Analyses: Each muscle sample was cut into 10 μm thick serial sections with a cryostat (-20°C). In one section, muscle fibres were classified as Type I (light staining) or Type II (dark staining) based on their staining intensity for myosin ATPase, alkaline pre-incubation (pH=9.4) (Nwyoye et al, 1982). Quantitative histochemical methods were used on subsequent serial sections to determine succinate dehydrogenase (SDH) and α-glycerophosphate dehydrogenase (GPD) enzyme activity according to the methods of Blanco et al. (1988) and Martin et al. (1985), respectively. Briefly, the SDH and GPD reactions were performed in 10.0 ml coplin jars as follows: SDH, 100 mM phosphate buffer, 10 μM sodium azide, 20 μM 1-methoxyphenazine methylsulphate, 1.5 mM nitro blue tetrazolium, 5 mM EDTA and 48 mM succinic acid, pH 7.6 for 8 minutes at room temperature; GPD, 100 mM phosphate buffer, 10 μM sodium azide, 20 μM 1-methoxyphenazine methylsulphate, 1.2 mM nitro blue tetrazolium, and 9.3 mM glycerophosphate, pH 7.4. in a water bath maintained at 37°C for 11 minutes. The sections were dried in the dark for 30 minutes before mounting with aquamount. Fibre cross-sectional area was determined from the muscle fibres stained for myosin ATPase. Muscle fibres were selected from a centrally located region within the cross-section of the soleus and plantaris. The tibialis anterior was divided into a deep (mixed type I and II) and superficial (exclusively type II) portions using connective tissue as a landmark. A sample of 40 - 50 fibres was taken from the soleus, plantaris and from each region of the tibialis anterior for analysis. The same 40 - 50 muscle fibres were identified in the serial sections prepared for ATPase, SDH and GPD and subsequently analyzed. The number of type I and II fibres analyzed was proportional to the fibre type composition of each muscle.

Statistics: Analysis of variance (ANOVA) was used to compare main effects of

GPA feeding and TTX-induced paralysis on mean body weight, tibial length, muscle wet weight, fibre type composition, ATP and PCr concentration of each muscle. Single fibre measurements of fibre area, SDH and GPD were used to generate type I and II fibre mean values for each muscle in C, GPA, TTX and GPA/TTX rats. Separate 2x2 factorial designs were used to analyze the fibre area, SDH and GPD data of type I and II fibres. An additional 2x2 factorial ANOVA was performed on the type II fibres from the superficial region of the tibialis anterior. Significance was accepted at $p < 0.05$. Newman-Keuls post-hoc comparison of means was used to test significance level set at 0.05.

4.3 Results

Muscle ATP and PCr: As reported elsewhere (Fitch et al., 1974, 1975), a significant decrease in ATP and PCr concentration was observed in each muscle following GPA feeding (Table IV-1). The concentration of ATP and PCr was also affected by TTX-induced paralysis. In the soleus, TTX-induced paralysis had no effect on ATP concentration in TTX rats, but significantly reduced it by 57% in GPA/TTX rats relative to GPA. In contrast, the concentration of PCr was decreased by 27% in TTX soleus by TTX-induced paralysis and was unaltered in GPA/TTX soleus (Figure IV-1). TTX-induced paralysis decreased ATP and PCr concentration in a similar manner in TTX and GPA/TTX plantaris (Table IV-1). The concentration of ATP in the tibialis anterior was decreased following TTX-induced paralysis in both TTX and GPA/TTX rats (Table IV-1). In contrast, PCr concentration was reduced by 59% in TTX tibialis anterior and was unaltered in GPA/TTX following TTX-induced paralysis. An increase in connective tissue following TTX-paralysed muscle would result in an underestimation of true ATP and PCr content. Although an effort was made to separate connective tissue from muscle, it is by no means certain that these means were entirely successful.

Muscle mass and fibre morphology: The mean wet weight of the soleus was significantly reduced following GPA feeding In addition, TTX-induced paralysis produced a marked atrophy of 48% in soleus wet weight (Table IV-2). The percent composition of type I fibres following GPA feeding was significantly increased in the soleus. In contrast, TTX-induced paralysis dramatically reduced the percentage of type I fibres by 20% in the soleus (Figure IV-1, Table IV-2). The cross-sectional area of type I fibres in the soleus was significantly reduced following GPA feeding, whereas, type II fibre area was unaltered (Table IV-3). In comparison, TTX-induced paralysis decreased cross-sectional area of both type I and II fibres of the soleus (Figure IV-2, Table IV-3). There was no interaction of main effects in either type I or type II fibres as a similar degree of atrophy occurred in type I (66 - 70%) and type II fibres (57 - 66%) of TTX and GPA/TTX soleus.

The mean wet weight of the plantaris was also significantly decreased by GPA feeding and TTX-induced paralysis to a similar extent as the soleus (Table IV-2). As in the soleus, there was no difference in muscle atrophy between TTX and GPA/TTX plantaris. In contrast to the soleus, GPA feeding did not increase the percentage of type I fibres in the plantaris. However, the percentage of type I fibres was significantly decreased from 17% to 10% following TTX-induced paralysis (Table IV-2). The fibre atrophy induced by GPA feeding in the plantaris differed from that which occurred in the soleus (Table IV-4). Instead of a decrease in cross-sectional area of type I fibres, the area of type II fibres was significantly reduced by 22%. As in the soleus, TTX-induced paralysis produced significant atrophy in both type I and II fibres of the plantaris. No interaction of GPA feeding and TTX-induced paralysis was evident as similar atrophy occurred in both TTX and GPA/TTX muscle fibres (Table IV-4).

As in the soleus and plantaris, GPA feeding and TTX-induced paralysis significantly decreased the mean wet weight of the tibialis anterior (Table IV-2). In the deep region of the tibialis anterior, GPA feeding increased the percentage of type I fibres. In contrast, TTX-induced paralysis decreased the percentage of type I fibres. The decrease in type I fibres following TTX-induced paralysis was significant in GPA/TTX muscles, but not in the tibialis anterior of TTX rats.

Comparable to the plantaris, another predominantly fast-twitch muscle, the type II fibres, and not type I fibres of the tibialis anterior atrophied following GPA feeding. The cross-sectional area of type II fibres from the deep and superficial regions of the tibialis anterior was reduced by 21% and 33%, respectively, following GPA feeding (Table IV-5). As in the soleus and plantaris, TTX-induced paralysis significantly reduced the cross-sectional area of type I and II fibres in the tibialis anterior. In contrast to the soleus and plantaris, the extent of atrophy in fibres of the superficial region of the tibialis anterior was greater in TTX rats than TTX/GPA rats. The cross-sectional area was decreased by 70% in TTX rats and by 60% in GPA/TTX rats.

Muscle fibre GPD and SDH enzyme activities: The effects of GPA feeding and TTX-induced paralysis on enzyme activity of the soleus are summarized in Table IV-3. In addition, single fibre enzyme measurements of representative soleus muscles are presented in Figure IV-3. The GPD activity of the soleus was unaltered in type I and II fibres following GPA feeding (Table IV-3). In contrast, TTX-induced paralysis increased GPD activity of type I fibres, but not type II fibres of the soleus. No interaction of GPA feeding and TTX-induced paralysis on GPD activity was present (Table IV-3). The SDH activity of the soleus was increased in type I fibres, but not type II fibres following GPA feeding. In contrast, TTX-induced paralysis significantly reduced SDH activity in type I and II fibres of the soleus. The effect of TTX on SDH activity was enhanced by GPA feeding in both type I and II fibres (Figure IV-6). For instance, type I fibre SDH activity was unaltered in TTX rats, but in GPA/TTX rats was decreased by 42% relative to GPA. The SDH activity of type II fibres was more susceptible to TTX-induced paralysis than type I fibres in both TTX and GPA/TTX rats as SDH activity was reduced by 41% and 56% relative to their respective controls (Figure IV-6).

Enzyme activities of type I and II fibres of the plantaris following GPA feeding and TTX-induced paralysis are summarized in Table IV-4. In addition, single fibre enzyme measurements of representative plantaris muscles are presented in Figure IV-4. In the plantaris, GPA feeding had no effect on GPD activity or on SDH activity of type I fibres, but significantly increased SDH activity of type II fibres (Table IV-4, Figure IV-4). TTX-induced paralysis significantly decreased GPD and SDH activity of the plantaris in both type I and II fibres (Table IV-4, Figure IV-4). The decrease in SDH activity of type I fibres was similar between TTX and GPA/TTX plantaris following TTX-induced paralysis. In contrast, the decrease in SDH activity in type II fibres was 34% in TTX rats and 50% in GPA/TTX rats. Thus, GPA feeding altered the response of type II fibre SDH activity to paralysis (Table IV-4, Figure IV-7).

The effects of GPA feeding and TTX-induced paralysis on enzyme activity of the tibialis anterior are summarized in Table IV-5. In addition, single fibre enzyme measurements of representative tibialis anterior muscles are presented in Figure IV-5. Adaptation of enzyme activity to GPA feeding and TTX-induced paralysis in the tibialis anterior depended on fibre type and muscle region. GPA feeding increased GPD activity of type I and type II fibres of the deep region, but superficial type II fibres were unaltered. Similarly, SDH activity was increased in type I and II fibres of the deep region, but was not significantly altered in the superficial region following GPA feeding (Table IV-5, Figure IV-5). TTX-induced paralysis significantly decreased GPD activity in deep and superficial type II fibres, but did not alter type I fibre GPD activity (Table IV-5, Figure IV-5). The GPD activity of deep region type II fibres was decreased to a greater extent in GPA/TTX rats (56%) than in TTX rats (32%) relative to GPA and C, respectively. The SDH activity in type I and II fibres of the tibialis anterior was decreased following TTX-induced paralysis (Table IV-5, Figure IV-5). The decrease in SDH activity in type II fibres of the superficial region was not different between TTX and GPA/TTX rats. In contrast, SDH activity in type I and II fibres of the deep region was decreased 45% - 55% in GPA/TTX rats and not at all in TTX rats (Figure IV-8).

4.4 Discussion

Changes in the intracellular concentrations of PCr, ATP and ATP phosphorylation potential have been associated with skeletal muscle adaptations during chronic electrical stimulation (Green et al., 1992) and chronic GPA feeding (Lai and Booth, 1990; Moerland et al., 1989). In each case, the expression of slow myosin heavy chain is increased, mitochondrial biogenesis is stimulated, oxidative enzyme activity increases and parvalbumin expression is decreased (Shoubridge et al., 1985; Pette and Vrbova, 1985; Moerland et al., 1989). The fundamental difference between the two perturbations is that GPA feeding decreases PCr and ATP concentration by pharmacological means whereas chronic electrical stimulation alters the activation pattern of the muscle which subsequently depresses the ATP phosphorylation potential. Prior to this study, it was unknown whether adaptations to GPA feeding occurred independently of neuromuscular activity or if neuromuscular activity was a required component.

Similar to previous reports (Shoubridge et al., 1985; Moerland et al., 1989; Lai and Booth, 1990; Shields et al., 1975), the present study observed preferential atrophy of type II fibres in predominantly fast-twitch muscle, an increase in the percent composition of type I fibres in the soleus and the deep region of the tibialis anterior and increased oxidative enzyme activity in type I and II fibres following GPA feeding. Contrary to Shoubridge et al. (1985), a decrease in cross-sectional area and an increase in SDH activity was observed in type I fibres of the soleus. The morphological and metabolic properties of the soleus, plantaris and tibialis anterior were dramatically affected by TTX-induced paralysis. As previously reported, type I fibre percent composition was decreased in the soleus (Spector, 1985). Contrary to two weeks of TTX-induced paralysis of plantaris and gastrocnemius (Michel and Gardiner, 1990; Gardiner et al., 1992), four weeks of paralysis in this study was sufficient to decrease the percentage of type I fibres in the plantaris and deep portion of the tibialis anterior. In addition, the marked fibre atrophy of type I and II fibres which occurred in the soleus (Spector, 1985), the plantaris (Michel and Gardiner, 1990) and the tibialis anterior was similar to previous reports

(St.-Pierre and Gardiner, 1985; 1990; Gardiner et al., 1992). As reported previously, the glycolytic enzyme activity of the soleus was increased by TTX (Wan and Boegman, 1981). In contrast, the glycolytic enzyme activity of fast-twitch muscles, in this case plantaris and tibialis anterior, was decreased (St.-Pierre et al., 1988; Gardiner et al., 1992). In every muscle examined, the SDH activity was decreased following TTX-induced paralysis. In corroboration of the present results, citrate synthase activity (St.-Pierre et al., 1988) and SDH activity (Gardiner et al., 1992) have been reported to decrease in the gastrocnemius following TTX-induced paralysis.

Unlike GPA feeding, where fibre-type specific and muscle specific effects were prevalent, TTX-induced paralysis generally had a similar effect on each muscle and on type I and II fibres. However, there were some exceptions. For instance, in only type I fibres of the soleus muscle was there a significant increase in GPD activity following TTX-induced paralysis. In all other muscles GPD activity was decreased. The extent of atrophy in type I and II fibres following TTX-induced paralysis was similar in the soleus and plantaris. In contrast, TTX-induced paralysis decreased cross-sectional area of type I and II fibres of the tibialis anterior to a varied extent. The fibre area of type II fibres from the deep and superficial region decreased by 25% and 66%, respectively, whereas type I fibre area decreased by only 19%. Clearly the cross-sectional area of superficial type II fibres was more susceptible to TTX-induced paralysis. This differs from the paralysed gastrocnemius in which type I fibre area decreased by 48% relative to 61% and 68% for deep and superficial type II fibres, respectively (Gardiner et al., 1992).

It was obvious from the results that the pharmacological depletion of ATP and PCr alone is not sufficient to maintain or alter muscle phenotype. The morphological and metabolic properties of GPA-fed muscles adapted to TTX-induced paralysis to a similar or greater extent in all but one instance. That one exception was a 60% reduction compared to a 70% reduction in type II fibre area of the superficial tibialis anterior. The two main effects of GPA feeding;

increased type I fibre percentage and increased SDH activity, were completely reversed by TTX-induced paralysis. The SDH activity in GPA-fed soleus, plantaris and tibialis anterior was actually reduced by a greater extent relative to C as was the type I fibre percentage in the deep tibialis anterior.

The ATP phosphorylation potential may play a role during adaptation to altered functional demand by stimulating gene expression and subsequent mitochondrial biogenesis (Green et al., 1992; Meerson, 1975). In this study, the pharmacological reduction of PCr and ATP stimulated phenotypic transformation and mitochondrial biogenesis. However, this adaptive stimulus was dependent on neuromuscular activity. Lai and Booth (1990) suggested that "exercise signals" are required in conjunction with GPA feeding to induce an increase in cytochrome c mRNA. Interestingly, TTX-induced paralysis decreased PCr concentration in each muscle examined and decreased ATP concentration in the plantaris and tibialis anterior. Therefore, the maintenance of ATP and PCr concentration may also be dependent on neuromuscular activity.

TABLE IV-1. Adenosine triphosphate and phosphocreatine concentrations.

Main effects	GPA		TTX	
	C	GPA	C	TTX
SOLEUS				
ATP	15.1 ± 0.7	7.0 ± 0.7[a]	12.0 ± 0.6	10.0 ± 0.8[b]
PCr	39.7 ± 1.3	6.5 ± 1.9[a]	25.6 ± 1.3	20.6 ± 1.9[ab]
PLANTARIS				
ATP	20.4 ± 0.9	11.4 ± 1.1[a]	18.4 ± 0.9	13.3 ± 1.1[a]
PCr	50.4 ± 3.3	6.2 ± 4.0[a]	32.4 ± 3.8	24.3 ± 3.5[a]
TIBIALIS ANTERIOR				
ATP	24.1 ± 1.1	11.1 ± 1.1[a]	23.6 ± 1.1	11.6 ± 1.1[a]
PCr	52.4 ± 2.9	3.6 ± 3.4[a]	39.4 ± 3.2	16.7 ± 3.2[ab]

Mean ± SEM. Main effects; GPA, rats fed β-guanidinopropionic acid; TTX, tetrodotoxin-induced paralysis. Left column; Control and TTX groups combined (C, n=18) vs. GPA and GPA/TTX groups combined (GPA, n=16). Right column; Control and GPA groups combined (C, n=18) vs. TTX and GPA/TTX groups combined (TTX, n=16). Abbreviations: ATP, adenosine triphosphate (μmol/mg dry weight); PCr, phosphocreatine (μmol/mg dry weight); [a], significant main effect; [b], significant interaction; $p < 0.05$.

TABLE IV-2. Muscle morphology.

Main effects	GPA		TTX	
	C	GPA	C	TTX
SOLEUS				
mg	95.9 ± 3.7	85.3 ± 3.1^{a}	119.0 ± 3.7	62.2 ± 3.1^{a}
%I	70 ± 2	75 ± 2^{a}	82 ± 2	62 ± 2^{a}
PLANTARIS				
mg	172.6 ± 6.0	149.6 ± 7.7^{a}	222.7 ± 8.2	99.5 ± 5.5^{a}
%I	13 ± 1	14 ± 1	17 ± 1	10 ± 1^{a}
TIBIALIS ANTERIOR				
mg	314.0 ± 12.9	278.2 ± 9.8^{a}	425.5 ± 13.1	166.8 ± 9.7^{a}
%I	5 ± 1	9 ± 1^{a}	9 ± 1	4 ± 1^{ab}

Mean ± SEM. Main effects; GPA, rats fed ß-guanidinopropionic acid; TTX, tetrodotoxin-induced paralysis. Left column; Control and TTX groups combined (C, n=18) vs. GPA and GPA/TTX groups combined (GPA, n=18). Right column; Control and GPA groups combined (C, n=20) vs. TTX and GPA/TTX groups combined (TTX, n=16). Abbreviations: mg, wet weight muscle mass; %I, percentage type I fibres; ^{a}, significant main effect, ^{b}, significant interaction; $p < 0.05$.

Table IV-3. Soleus single fibre area and enzyme activities.

Main effect	GPA		TTX	
	C	GPA	C	TTX
Type I Fibres				
CSA	1433 ± 58	1233 ± 55[a]	2015 ± 52	651 ± 60[a]
GPD	2.5 ± 0.5	3.0 ± 0.5	2.0 ± 0.4	3.4 ± 0.5[a]
SDH	20.4 ± 1.4	25.4 ± 1.4[a]	27.5 ± 1.3	18.4 ± 1.5[ab]
Type II Fibres				
CSA	776 ± 44	717 ± 46	1077 ± 40	416 ± 46[a]
GPD	4.0 ± 0.5	4.1 ± 0.5	3.4 ± 0.5	4.7 ± 0.6
SDH	26.3 ± 1.7	29.8 ± 1.6	37.3 ± 1.5	18.9 ± 1.8[ab]

Mean ± SEM. Main effects; GPA, rats fed β-guanidinopropionic acid; TTX, tetrodotoxin-induced paralysis. Left column; Control and TTX groups combined (C, n=18) vs. GPA and GPA/TTX groups combined (GPA, n=18). Right column; Control and GPA groups combined (C, n=20) vs. TTX and GPA/TTX groups combined (TTX, n=16). Abbreviations: CSA, cross-sectional area (μm^2); GPD, α-glycerophosphate dehydrogenase activity ((OD/min) x 10^3); SDH, succinate dehydrogenase activity ((OD/min) x 10^3); [a], significant main effect; [b], significant interaction; $p < 0.05$.

Table IV-4. Plantaris single fibre area and enzyme activities.

Main effect	GPA		TTX	
	C	GPA	C	TTX
Type I Fibres				
CSA	1436 ± 77	1291 ± 77	1914 ± 72	812 ± 81[a]
GPD	4.3 ± 0.6	5.4 ± 0.6	5.9 ± 0.6	3.8 ± 0.6[a]
SDH	30.2 ± 1.8	34.3 ± 1.8	37.9 ± 1.7	26.6 ± 1.9[a]
Type II Fibres				
CSA	1096 ± 51	838 ± 51[a]	1408 ± 48	526 ± 54[a]
GPD	7.0 ± 0.7	8.2 ± 0.7	9.7 ± 0.7	5.4 ± 0.8[a]
SDH	30.1 ± 1.9	36.8 ± 1.9[a]	42.7 ± 1.8	24.2 ± 2.0[ab]

Mean ± SEM. Main effects; GPA, rats fed ß-guanidinopropionic acid; TTX, tetrodotoxin-induced paralysis. Left column; Control and TTX groups combined (C, n=18) vs. GPA and GPA/TTX groups combined (GPA, n=18). Right column; Control and GPA groups combined (C, n=20) vs. TTX and GPA/TTX groups combined (TTX, n=16). Abbreviations: CSA, cross-sectional area (μm^2); GPD, α-glycerophosphate dehydrogenase activity ((OD/min) x 10^3); SDH, succinate dehydrogenase activity ((OD/min) x 10^3); [a], significant main effect; [b], significant interaction; $p < 0.05$.

Table IV-5. Tibialis anterior single fibre area and enzyme activities.

Main effect	GPA		TTX	
	C	GPA	C	TTX
Type I Fibres				
CSA	1235 ± 70	1077 ± 69	1280 ± 65	1032 ± 74[a]
GPD	4.2 ± 0.5	5.5 ± 0.4[a]	5.2 ± 0.4	4.4 ± 0.5
SDH	30.4 ± 1.9	37.7 ± 1.8[a]	40.4 ± 1.7	27.6 ± 2.0[ab]
Deep Type II Fibres				
CSA	981 ± 52	771 ± 51[a]	1004 ± 48	748 ± 54[a]
GPD	6.4 ± 0.7	8.3 ± 0.6[a]	9.6 ± 0.6	5.1 ± 0.7[ab]
SDH	35.3 ± 2.3	44.3 ± 2.3[a]	49.9 ± 2.2	29.7 ± 2.4[ab]
Superficial Type II Fibres				
CSA	1416 ± 99	947 ± 105[a]	1765 ± 92	598 ± 111[ab]
GPD	9.5 ± 0.9	9.0 ± 0.9	11.2 ± 0.8	7.4 ± 1.0[a]
SDH	22.4 ± 1.9	25.0 ± 2.0	26.8 ± 1.7	20.5 ± 2.1[a]

Mean ± SEM. Main effects; GPA, rats fed ß-guanidinopropionic acid; TTX, tetrodotoxin-induced paralysis. Left column; Control and TTX groups combined (C, n=20) vs. GPA and GPA/TTX groups combined (GPA, n=20). Right column; Control and GPA groups combined (C, n=24) vs. SA and GPA/SA groups combined (SA, n=16). Abbreviations: CSA, cross-sectional area (μm^2); GPD, α-glycerophosphate dehydrogenase activity ((OD/min) x 10^3); SDH, succinate dehydrogenase activity ((OD/min) x 10^3); [a], significant main effect; [b], significant interaction; $p < 0.05$.

Figure IV-1. Myofibrillar ATPase staining reaction of representative soleus muscles from control (C), TTX (CX), GPA-fed (A) and GPA/TTX (AX) rats. Type II fibres are darkly stained. See methods section for details. Tissue was photographed at magnification of 10x with subsequent 2x enlargement of prints.

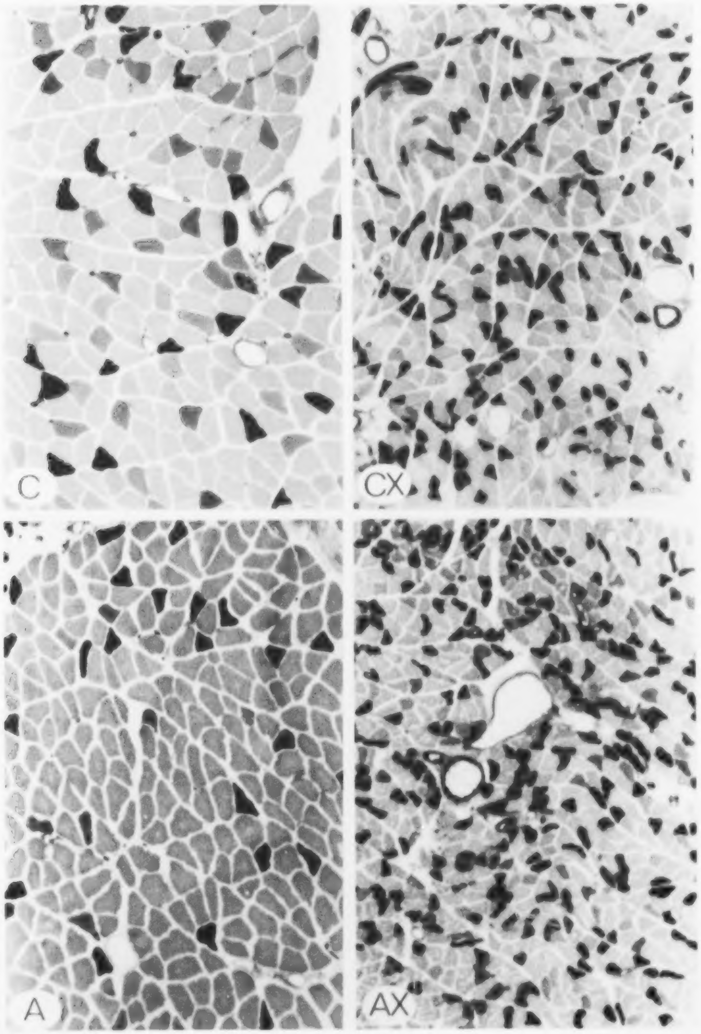


FIGURE IV–2. Single fibre measurements of enzyme activity in representative soleus muscles.
○ Type I fibres; ● Type II fibres

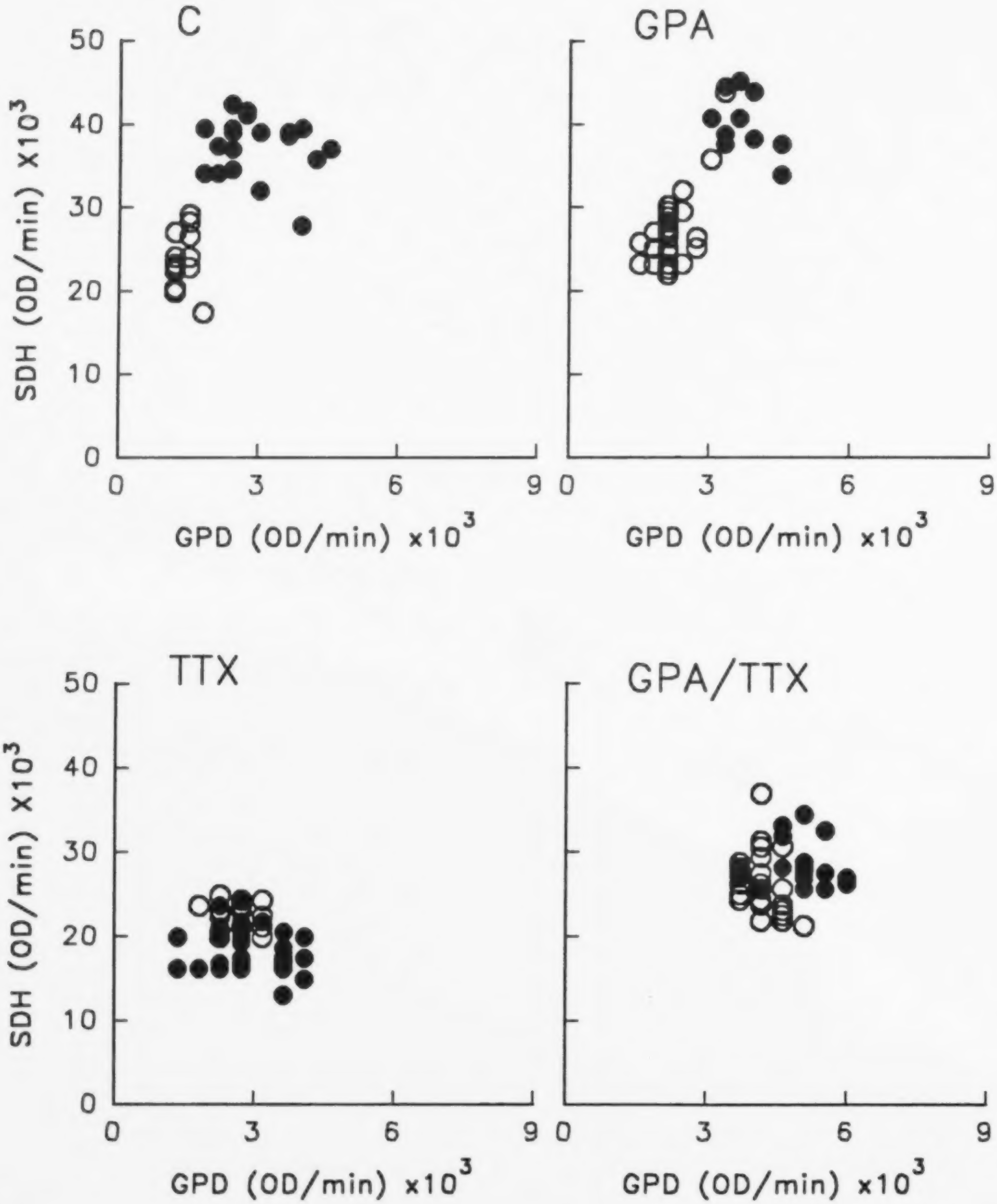


FIGURE IV–3. Single fibre measurements of enzyme activity in representative plantaris muscles. ○ Type I fibres; ● Type II fibres.

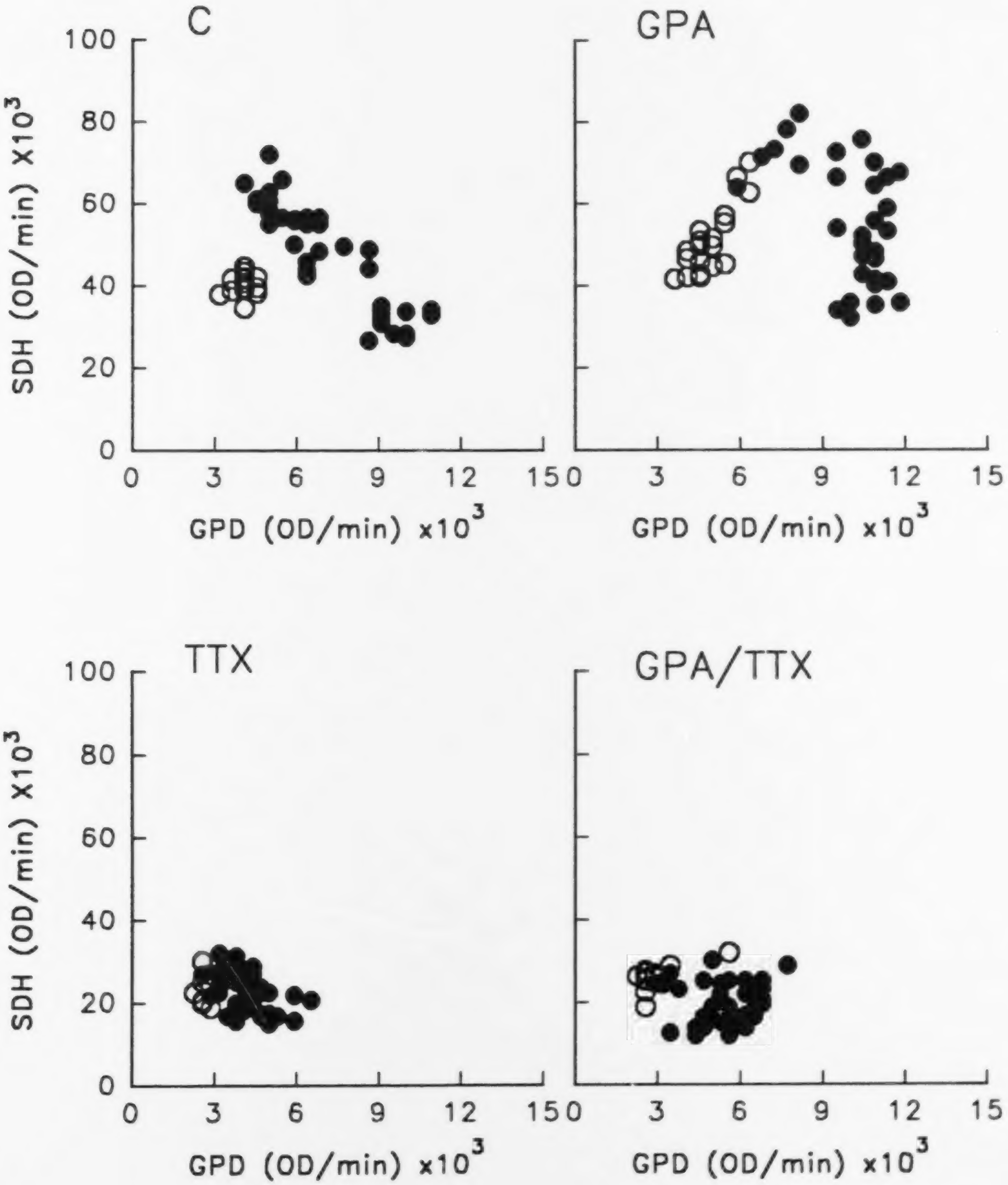


FIGURE IV–4. Single fibre measurements of enzyme activity in representative deep and superficial regions of the tibialis ant. ○ Type I fibres, ● , ■ Type II fibres of the deep and superficial regions, respectively.

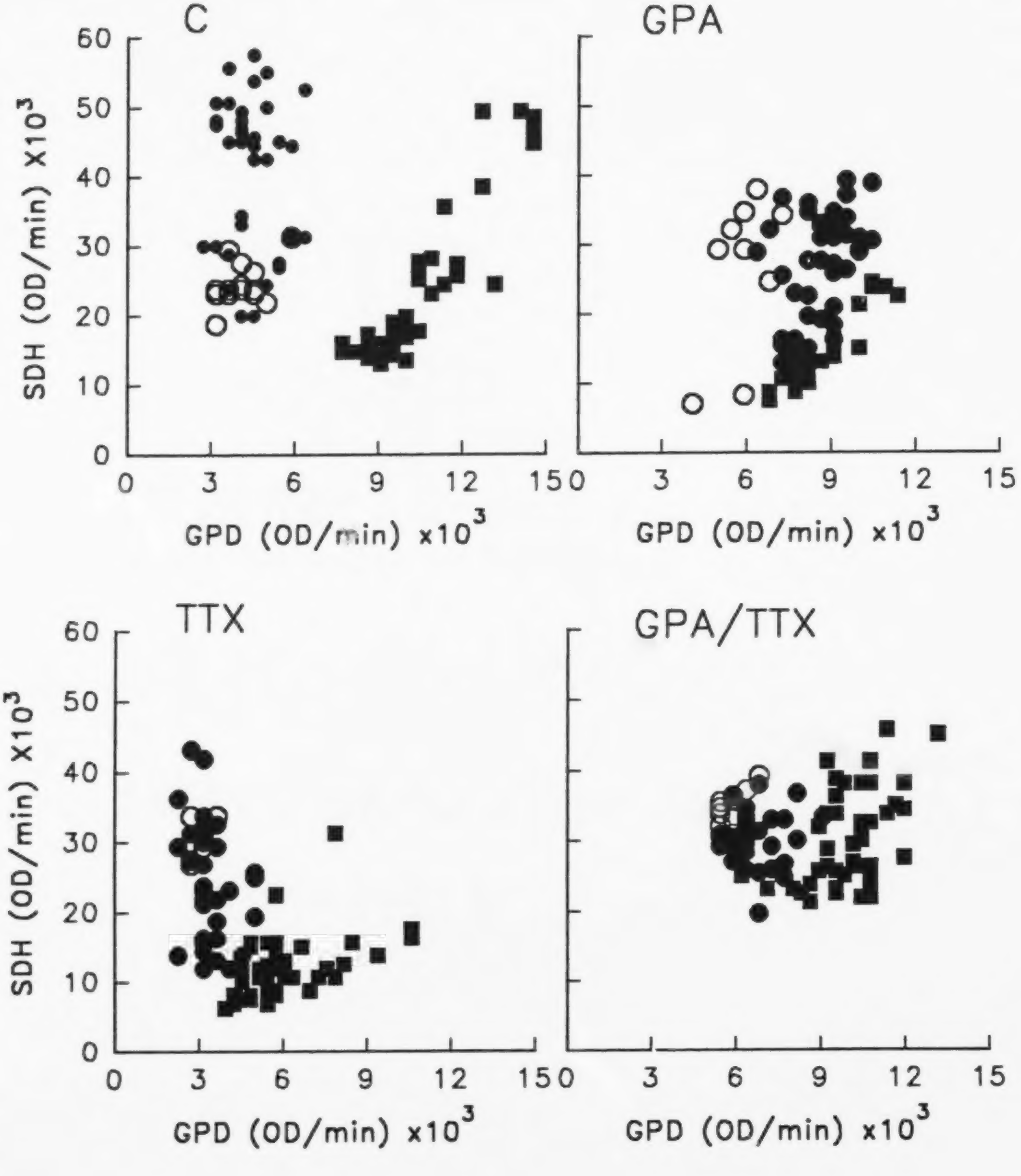


FIGURE IV–5. Soleus SDH activity in type I and II fibres of control, GPA–fed, TTX–paralyzed (TTX)nd and GPA–fed combined with TTX–induced paralysis (GPA/TTX). Different letters on top of bars indicates significant differences, $p < 0.05$.

□ control rats. ■ GPA–fed rats.

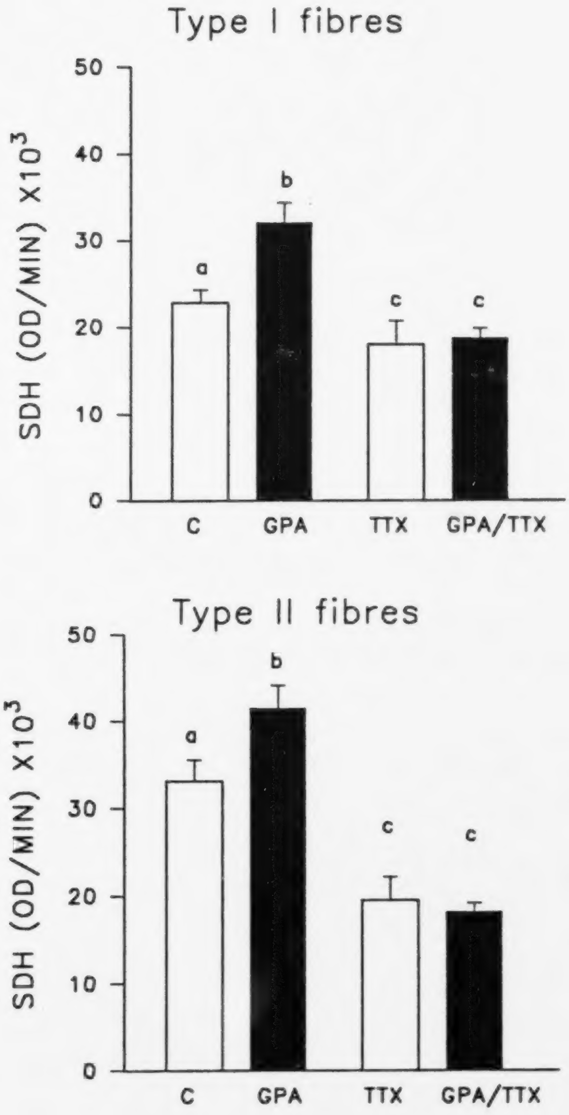


FIGURE IV–6. Plantaris SDH activity in type I and II fibres of control, GPA–fed, TTX–paralyzed (TTX)nd and GPA–fed combined with TTX–induced paralysis (GPA/TTX). Different letters on top of bars indicates significant differences, $p < 0.05$.

☐ control rats. ■ GPA–fed rats.

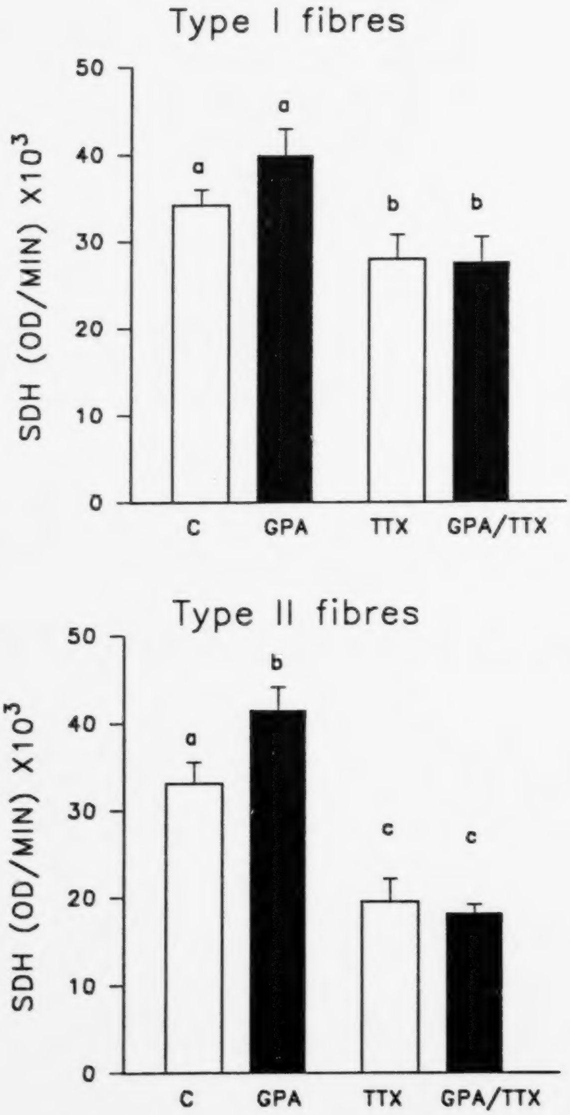
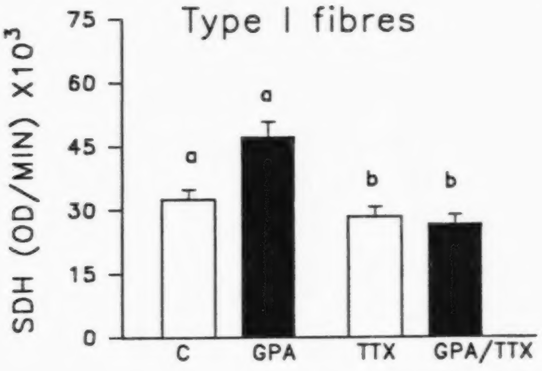
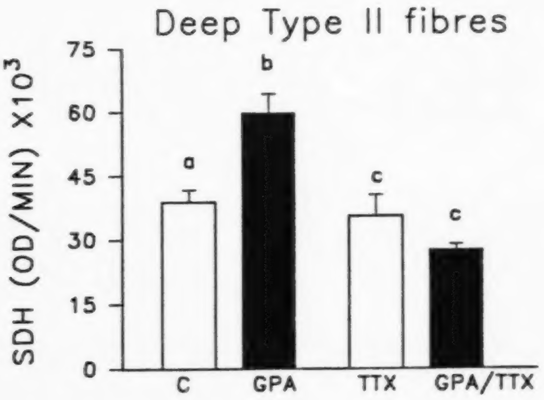
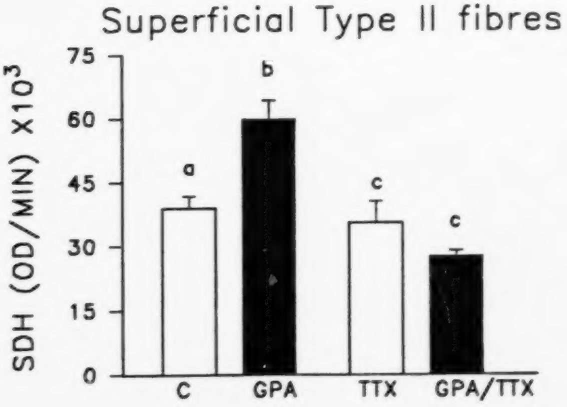


FIGURE IV–7. Tibialis Ant. SDH activity in type I and II fibres of control, GPA–fed, TTX–paralyzed (TTX) and GPA–fed combined with TTX–induced paralysis (GPA/TTX). Different letters on bars denote significant differences $p < 0.05$.
☐ control rats. ■ GPA–fed rats.


Type I fibres


Deep Type II fibres


Superficial Type II fibres

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CHAPTER V

Adaptation of skeletal muscle to tetrodotoxin-induced paralysis in normal and ß-guanidinopropionic acid fed rats: 2. Effect on protein turnover.[4]

5.1 Introduction

At any given time a cell's complement of protein is dependent on the balance of transcription of the genetic code, translation of mRNA to synthesize proteins and proteolysis of existing proteins. The product of these processes determine the morphological, contractile and metabolic properties of skeletal muscle. It is the capacity of skeletal muscle to alter its protein complement in response to functional demand, environmental factors, and disease that has made it a subject of scientific scrutiny. Multiple sites of regulation, including pretranslational, translational and posttranslational mechanisms, are activated to adapt skeletal muscle to increased or decreased functional demands (Booth and Kirby, 1992). In addition, protein synthesis and degradation are two separate cellular processes which are precisely regulated and are capable of changing independently of one another.

In the previous chapter two perturbations; GPA feeding and tetrodotoxin (TTX)-induced paralysis, were examined. In each case the changes in muscle wet weight, fibre type composition and enzyme activities indicate that the rate of synthesis and/or degradation of protein has been altered. The rate of protein synthesis (Walton and Gill, 1978) and some protein degradative systems are energy dependent (Gronostaski et al., 1985; Fagan et al., 1992). Therefore, it is of interest to investigate the in vivo rates of protein synthesis and degradation in an animal that has adapted to a chronic reduction in the intramuscular concentration of PCr and ATP. In Chapter III it was concluded that GPA feeding limited mitochondrial biogenesis following synergist ablation. In Chapter IV, the adaptation of GPA-fed muscle to TTX-induced paralysis was equal to and in some instances, greater than control muscle. Aside from behavioral modifications, the only means of adaptation to these functional and/or pharmacological perturbations is degradation and synthesis of proteins. Therefore, an examination of the rates of protein synthesis and degradation in the soleus, plantaris and tibialis anterior was essential.

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[4] Co-authors include V. E. Baracos - discussion and editing of manuscript

As in the preceding chapters, the soleus, plantaris and tibialis anterior muscles were chosen for analysis because they vary in function, fibre type composition and metabolic profile. Skeletal muscles classified as predominantly slow-twitch or fast-twitch differ in their inherent rates of protein synthesis. Generally, slow-twitch muscles have a higher rate of protein synthesis than muscles with a high percentage of fast-twitch fibres (Garlick et al., 1989). A strong positive correlation between a muscle's percent area of slow oxidative fibres and the rate of protein synthesis has been reported (Garlick et al., 1989). Garlick et al. (1989) attributed the relationship to contraction speed and not to relative oxidative capacity. Measurements of protein turnover in control and GPA-fed muscle presents an opportunity to examine the relationships between the rates of protein synthesis to oxidative capacity, fibre-type composition and the concentration of ATP and PCr. In addition, how these variables affect protein turnover and subsequent adaptation to TTX-induced paralysis will also be examined.

5.2 Methods

Animals and experimental design: Studies were conducted in accordance with the Guidelines of the Canadian Council on Animal Care. Female Sprague-Dawley rats, 50 days old were obtained from a colony maintained in the Department of Animal Sciences, University of Alberta. Rats were housed 2 per cage and were maintained at 26°C on a 12 hour light/dark cycle. Animals were weight-matched and divided into four groups; C, control; TTX, TTX-induced paralysis; GPA, diet supplemented with 2% ß-guanidinopropionic acid;

GPA/TTX, TTX-induced paralysis combined with the GPA diet. The C, TTX (regular rat chow), GPA and GPA/TTX rats (GPA diet) were fed ad libitum. The body weight of each rat was measured weekly. At the end of 6 weeks C and GPA animals were sacrificed (n=6). The TTX and GPA/TTX rats were anaesthetized by inhalation of Metafane and an osmotic pump (Alza Corp., model 2ML4), silastic catheter and nerve cuff were implanted subcutaneously according to St. Pierre and Gardiner (1985). The TTX (80 μg/ml) was delivered at a constant rate (0.5 μl/hr) perfusing the sciatic nerve for 7 days. Neuromuscular paralysis was confirmed by assessing the absence of the grasping reflex in the afflicted limb. A minimum of 6 days confirmed absence of reflex was required for inclusion in subsequent analyses. All muscles from limbs successfully paralyzed were designated as TTX or GPA/TTX. The undisturbed contralateral limb muscles of these rats were compared with C and GPA muscle results; if no differences existed then the results were pooled.

Measurement of in vivo protein turnover: Protein synthesis was determined using the flooding-dose method of Garlick et al. (1980). On the day of the experiment between the hours of 1000 and 1200, rats were given an intraperitoneal injection of 150 μmoles phenylalanine (Phe) containing 50 μCi L-(2,6-^{3}H) Phe (Amersham, U.S.A.) in 1.5 mL of sterile phosphate buffered saline per 100 g body weight. After precisely 20 minutes, rats were stunned by CO_2 gas and killed by cervical dislocation. The rat carcass was immediately immersed in ice-cold water and the hind limbs skinned. The tibialis anterior, plantaris and soleus muscles were then removed from each limb, blotted dry and frozen in liquid nitrogen. All muscles were removed and frozen in less than 3 minutes. Muscles were stored at -50°C until analyzed.

The specific radioactivity of free (S_A) and protein bound (S_B) Phe was measured for each muscle. The fractional rate of protein synthesis (k_{syn}) was calculated as %/day from the following formula: $k_{syn} = (S_B \times 100)/S_A \times t$, where t is the time in days between injection and sacrifice. The difference in mean Phe mass between the muscles of C and GPA rats and the muscles from TTX and GPA/TTX rats was used to calculate k_{growth} for C, TTX, GPA and GPA/TTX rats over that 7 day period. The fractional degradation rate (k_{deg}) for each muscle was calculated by subtracting fractional growth rate (k_{growth}) from k_{syn}. As the mean growth and synthesis rates were used to calculate degradation for each group, no statistical analysis of this variable was possible. The absolute rate of protein synthesis (SYN) and degradation (DEG) were calculated by multiplying the fractional rates by the Phe mass.

Analysis of protein synthesis: Tissue samples were prepared in a similar manner to that reported by Garlick et al. (1980). Entire muscles were homogenized with approximately 1.5 - 3 mL ice-cold 2% $HClO_4$, depending on the size of muscle, using a motorized ground glass tissue homogenizer. Samples were then centrifuged for 15 minutes at 200 g and 0°C. Free and protein bound Phe specific radioactivity were measured after transformation to ß-phenylethylamine as described previously by Baracos et al. (1991). Phe mass was measured by HPLC according to the method of Jones and Gilligan (1983).

Protein synthesis relationships: Data from the preceding paper was used to determine the relationship of protein synthesis to ATP concentration, type I fibre mass percent composition and average SDH activity. The mean percent area occupied by type I fibre mass in each muscle was estimated by multiplying the mean cross-sectional area of type I and II fibres by their percentage frequency to obtain a total and expressing type I mass as a percentage of the total. Average muscle SDH activity was estimated as the sum of type I and II SDH activities multiplied by their respective fibre mass percent composition. The relative contribution of deep and superficial regions of the tibialis anterior to total mass was 20% and 80%, respectively (Armstrong and Phelps, 1984).

Statistics: The effects of treatment on each muscle were tested using multifactorial ANOVA. When significant main effects or interaction were determined ($p < 0.05$) differences between means were assessed with Newman-Keuls post-hoc analysis at $p < 0.05$. The relationships between protein synthesis, ATP concentration, fibre mass percent composition and mean

SDH activity following GPA feeding and TTX-induced paralysis was determined by correlation analysis. Results are reported in the text and tabulated as means ± standard error of the mean.

5.3 Results

Initial body weight of the rats at age 50 days was 150 g. After six weeks, body mass was significantly different between C (203 ± 2 g) and GPA (191 ± 3 g) rats. The difference in Phe mass between 6 weeks and 7 weeks control muscles indicated growth rates that were not significantly different from zero in the tibialis anterior, plantaris or soleus muscles. Therefore, muscle protein turnover was in steady state (ie. $k_{syn} = k_{deg}$). This was also true for the tibialis anterior and soleus of the GPA-fed rats, but not the plantaris which exhibited a k_{growth} of 1%/day. Chronic ingestion of GPA significantly reduced Phe mass in each muscle examined (Figure V-1). The sensitivity of Phe mass to GPA feeding was muscle dependent, as Phe mass was decreased by 27% and 31% in the plantaris and tibialis anterior of GPA rats, respectively, whereas soleus Phe mass decreased only 14% (Figure V-1). TTX-induced paralysis also significantly reduced Phe mass in each muscle. The Phe mass of TTX soleus, plantaris and tibialis anterior was reduced by 36%, 20%, and 13%, respectively (Figure V-1). There was no difference in the loss of Phe mass between C and GPA-fed muscle following TTX-induced paralysis.

Protein synthesis and degradation: As reported previously (Garlick et al., 1989), the fractional rate of protein synthesis differed significantly between the soleus, plantaris and tibialis anterior (Figure V-2). The highest rate of k_{syn} was observed in the soleus, whereas the tibialis anterior had the lowest. In the soleus, GPA feeding had no effect on k_{syn} or k_{deg} as both values were similar to those seen in controls. In contrast, TTX-induced paralysis decreased k_{syn} in the soleus by 56%, but had no effect on k_{deg} (Figure V-2). In the plantaris, k_{syn} was increased by 54% following GPA feeding, whereas k_{deg} was unaltered. After 1 week of TTX-induced paralysis, k_{syn} was decreased by 15% in the plantaris (Figure V-2). TTX-induced paralysis also increased k_{deg} in the plantaris of TTX (27%) and

GPA/TTX (66%) rats relative to C and GPA rats, respectively. Comparable to the plantaris, GPA feeding increased k_{syn} in the GPA rat tibialis anterior by 29%, but in contrast to the plantaris k_{deg} was also increased in GPA tibialis anterior by 38% relative to C. TTX-induced paralysis significantly increased k_{syn} by 8%. The k_{deg} of both TTX and GPA/TTX tibialis anterior were increased by 59% and 32%, relative to C and GPA, respectively, following TTX-induced paralysis. In no instance did the rate of protein synthesis of GPA-fed muscle and C muscle differ in their response to TTX-induced paralysis

In summary, the effect of GPA feeding and TTX-induced paralysis differed between the predominantly slow-twitch soleus and the predominantly fast-twitch plantaris and tibialis anterior. GPA feeding did not alter either protein synthesis or protein degradation in the soleus, but increased protein synthesis in the plantaris and tibialis anterior. The primary effect of TTX-induced paralysis on protein turnover differed between the predominantly fast-twitch muscles, plantaris and tibialis anterior, wherein protein degradation was increased and the slow-twitch soleus in which protein synthesis was decreased.

5.4 Discussion

The pharmacological effects of GPA feeding occur slowly over a period of weeks. The concentration of PCr and ATP are progressively decreased which may preclude any acute effects that decreased concentrations of PCr and ATP may otherwise have had on protein synthesis and degradation. At six weeks adaptations have occurred such that protein synthesis and degradation in GPA-fed muscle are in steady state. This point is corroborated by the fact that growth rates were not significantly different between muscles of six and seven week GPA-fed rats. Therefore, it is difficult to compare the observed increase in the rate of protein synthesis in the plantaris and tibialis anterior following GPA feeding with previous reports of decreased protein synthesis rates following acute changes in cell energy status. For instance, immediately following in situ fast-twitch muscle contraction a decrease in protein synthesis was observed concomitant with 30-50% and 80% decreases in ATP and PCr concentrations, respectively (Bylund-Fellenius et al., 1984). The results from in vitro preparations of Escherichia coli (St. John and Goldberg, 1978), growing fibroblasts (Gronostajski et al., 1985) and tumor cell lysates (Hucul et al., 1985) have also indicated that the rate of protein synthesis and degradation is dependent on the energy status of the cell. In each case, a decrease in ATP or ATP:ADP ratio was associated with a decrease in protein synthesis. Thus, a chronic reduction of ATP and PCr in skeletal muscle differs from an acute in situ or in vitro depletion of ATP as protein synthesis was increased or unchanged following GPA feeding. Furthermore, ATP concentration and the rate of protein synthesis were inversely correlated ($r=-0.674$) when analyzed in the soleus, plantaris and tibialis anterior muscles of control and GPA-fed rats (Figure V-3a).

Protein turnover was altered by GPA feeding and TTX-induced paralysis in a muscle specific manner. Following GPA feeding and subsequent decrease in the concentrations of ATP and PCr, the predominantly fast-twitch muscles, plantaris and tibialis anterior, exhibited an increased rate of protein synthesis. In contrast, there was no change in the predominantly slow-twitch soleus (Figure V-2). The three muscles also varied in their susceptibility to paralysis-induced atrophy as protein-bound Phe mass was decreased by 33% in the soleus, relative to 19% and 14% in the plantaris and tibialis anterior, respectively. Similar to the gross atrophy of muscle wet weight (Chapter IV), the decreases in protein-bound Phe mass was similar between GPA-fed and control muscles. The disuse atrophy of muscle has been linked to increased calcium-activated proteases (Ellis and Nagainis, 1984) and increases in the specific activity of cathepsin B and D proteases (Goldspink et al., 1986). In the present study, protein degradation appeared to increase in paralysed tibialis anterior and plantaris, but not in the soleus. Furthermore, a depletion of ATP has been reported to stimulate calcium-activated proteases and enhance protein degradation in incubated chick skeletal muscle (Fagan et al., 1992). In addition, protein degradation was higher in the tibialis anterior and plantaris, but not in the soleus of GPA-fed rats relative to control. Thus, it seems that the effect of TTX-induced paralysis and energy depletion on protein degradation is muscle dependent.

It has been suggested that the type I fibre mass percent composition is a major determinant of the muscle's rate of protein synthesis (Garlick et al., 1989). For instance, soleus and adductor longus are predominantly composed of type I fibres and have a higher rate of protein synthesis than the predominantly type II tibialis anterior. In contrast, the variability in rates of protein synthesis of five fast-twitch muscles could not be accounted for by differences in percent composition of fast-oxidative fibres. From this data, Garlick et al. (1989) suggested that the relative oxidative capacity of muscles did not account for the varied rates of protein synthesis, but that speed of contraction was the important factor. A comparison of protein synthesis rates and SDH activity across GPA-fed and TTX-paralysed muscles revealed only a weak correlation ($r=0.586$) between SDH activity and rate of protein synthesis. In addition, there was no correlation between type I fibre mass percent composition and protein synthesis. When paralysed muscles were removed from the analysis and only control and GPA-fed muscles included, protein synthesis was correlated ($r=0.834$) with type I fibre mass percent composition (Figure V-3b). Differences in the relationship of protein synthesis and oxidative capacity or fibre type composition between the predominantly fast-twitch muscles and the soleus were apparent. In the soleus, the type I fibre mass percent composition was strongly correlated ($r=0.838$) and SDH activity weakly correlated ($r=0.558$) with protein synthesis rate (Figure V-4a,b). In contrast, there was no correlation between protein synthesis and SDH activity or type I fibre mass percent composition in the plantaris or the tibialis anterior (Figure V-5a,b). Therefore, in corroboration of Garlick et al. (1989), we observed that when muscle protein turnover is in a steady state the rate of protein synthesis is correlated with slow myosin content, but not oxidative capacity. However, when muscle is in a catabolic state, protein synthesis was not related to slow myosin content or oxidative capacity.

In summary, the response of in vivo protein synthesis and degradation to GPA feeding and TTX-induced paralysis differed between the slow-twitch soleus and the predominantly fast-twitch plantaris and tibialis anterior. The relationships of protein synthesis to oxidative capacity and fibre type composition in the soleus and the lack thereof in plantaris and tibialis anterior, imply that the regulation of translational mechanisms may differ between predominantly slow- and fast-twitch muscles. The predominant effect of TTX-induced paralysis, regardless of prior diet, was dramatic atrophy of muscle protein. In the soleus, loss of protein could be attributed to a decreased rate of protein synthesis. In contrast, atrophy in the fast-twitch muscles was primarily due to an increased rate of protein degradation.

FIGURE V-1. Protein-bound phenylalanine mass in the tibialis ant., plantaris and the soleus.
a, significant main effect of GPA or TTX.
□ C + TTX ■ GPA + GPA/TTX
▨ C + GPA ▩ TTX + GPA/TTX

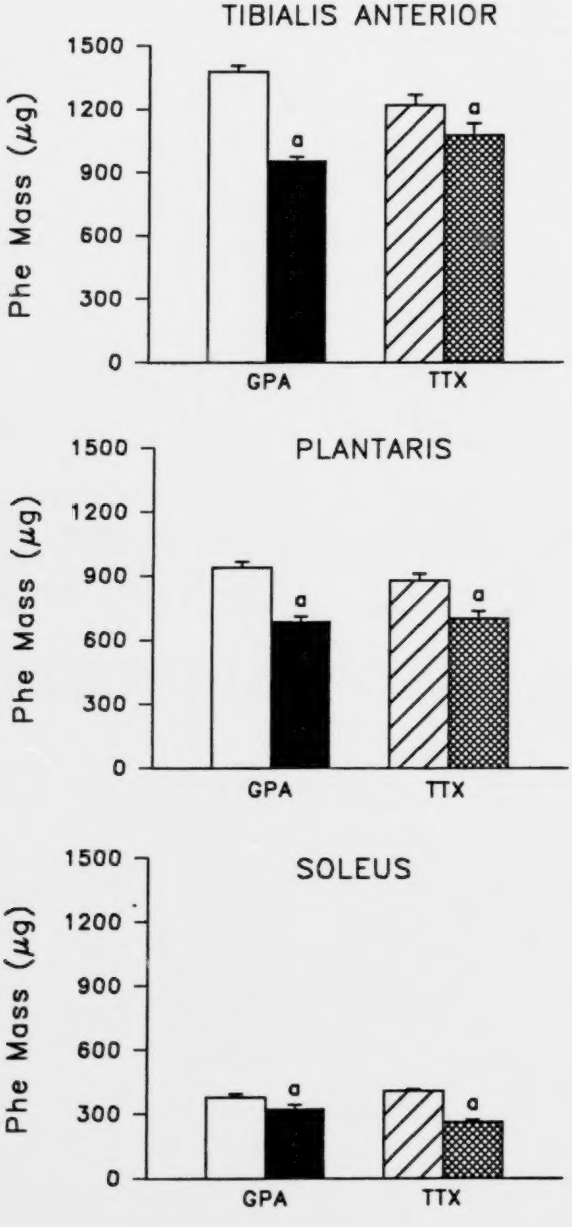


FIGURE V–2. Protein turnover in control, GPA–fed and paralyzed tibialis ant., plantaris and soleus.

a, significant main effect of GPA or TTX.

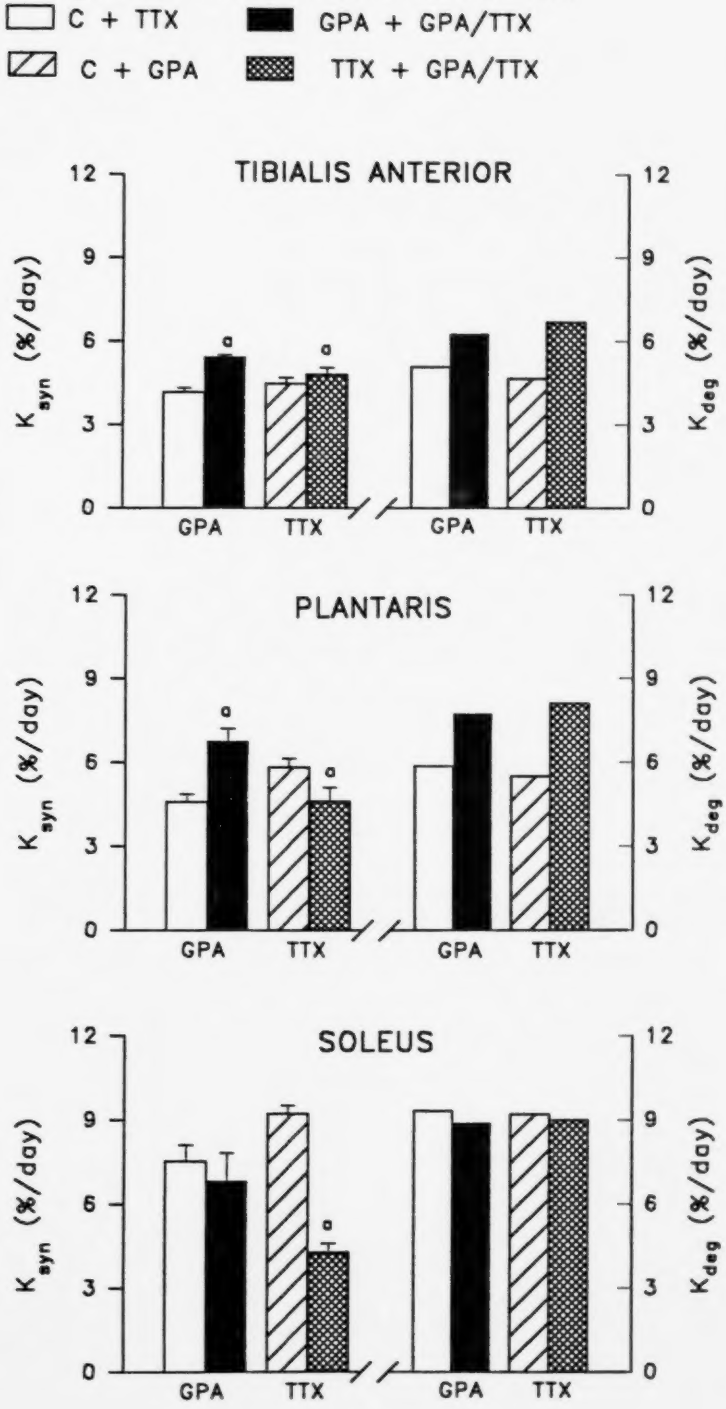


FIGURE V-3. Absolute rate of protein synthesis in the tibialis ant., plantaris and the soleus. a, significant main effect of GPA or TTX.

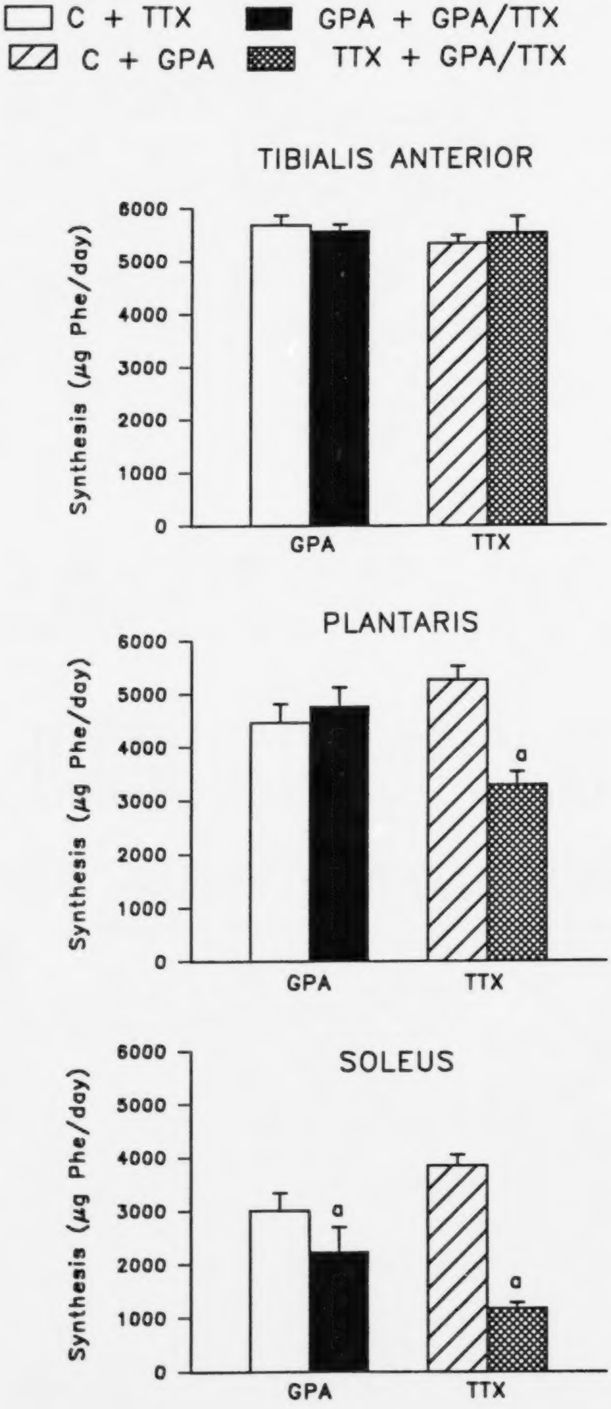


FIGURE V–4. Correlation between ATP concentration (A) and percentage of type I fibres (B) to protein synthesis rate in control and GPA–fed muscles.
● Control; ○ GPA

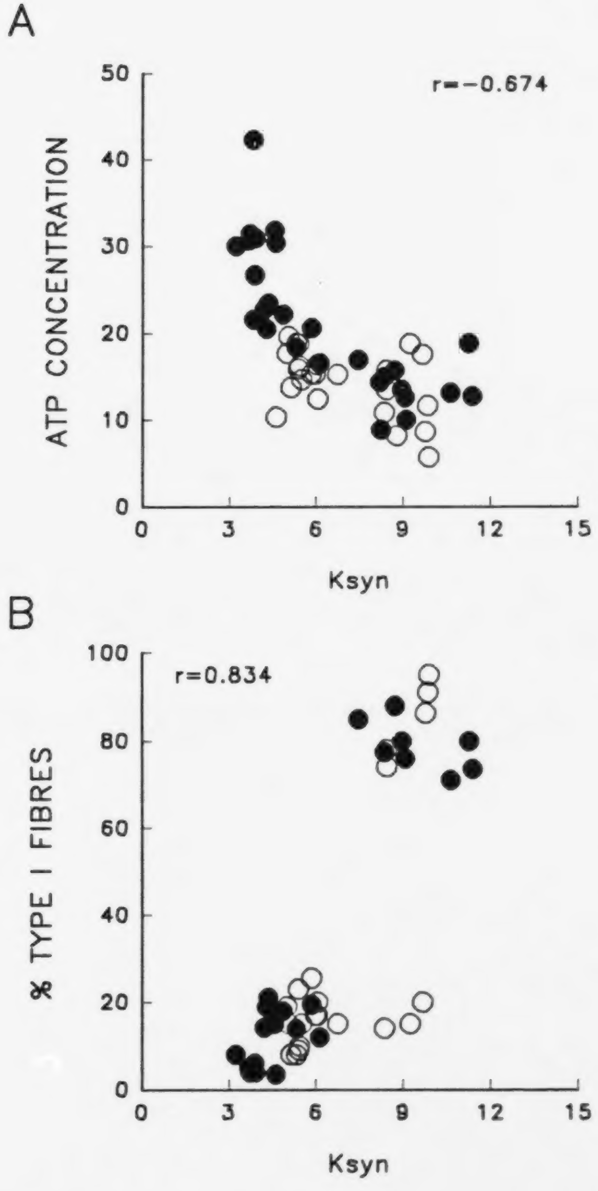


FIGURE V–5. Protein synthesis relationships following TTX–induced paralysis in C and GPA–fed soleus. A. protein synthesis and SDH activity. B. protein synthesis and fibre type.

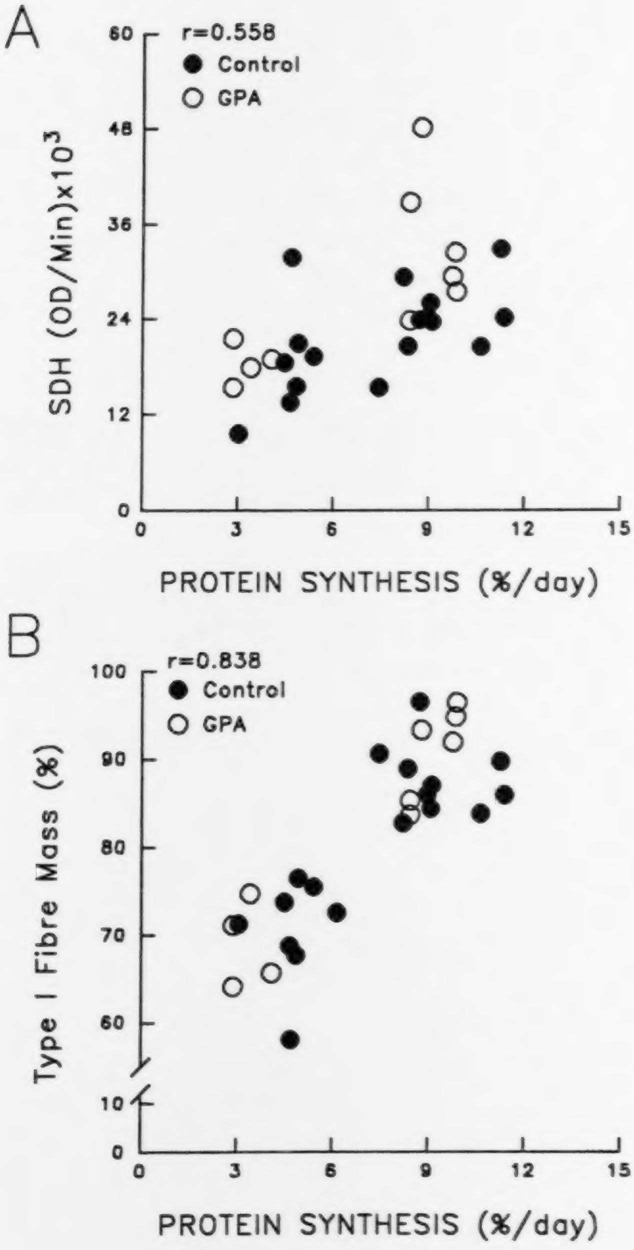
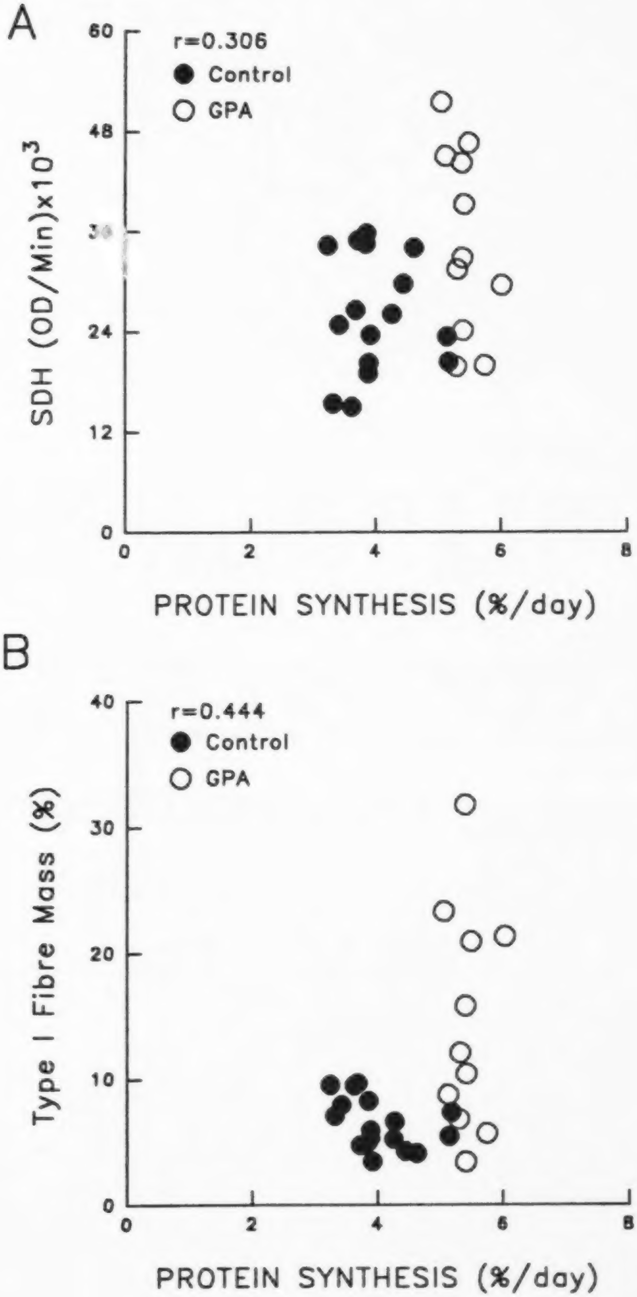


FIGURE V–6. Protein synthesis relationships following TTX–induced paralysis in C and GPA–fed tibialis ant. A. protein synthesis and SDH activity. B. protein synthesis and fibre type.



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CHAPTER VI

GENERAL DISCUSSION and CONCLUSIONS

The replacement of phosphocreatine (PCr) with the creatine analogue ß-guanidinopropionic acid (GPA) and the subsequent metabolic and morphological adaptations in skeletal muscle formed the basis of this thesis. Morphological and metabolic adaptations consistent with previous reports (Shoubridge et al., 1985; Lai and Booth, 1990) were apparent in the soleus, plantaris and tibialis anterior muscles of rats which ingested GPA over a period of eight weeks. An increased succinate dehydrogenase (SDH) activity in type I and II fibres of each muscle was the predominant effect. The tibialis anterior was the muscle most responsive to GPA feeding. In addition to an increased SDH activity following GPA feeding, α-glycerophosphate dehydrogenase activity was increased and fibre type specific atrophy occurred. The type II fibres of the superficial region of the tibialis anterior atrophied more than type I or II fibres of the deep region. This is similar to previous reports on the GPA-fed gastrocnemius in which atrophy was more extensive in the superficial region especially in type IIb fibres (Shields et al., 1975; Shoubridge et al., 1985).

Superimposing functional perturbations on rat muscle adapting to GPA feeding constituted the novel aspect of this research project. The purpose of the functional perturbations was to test the importance of normal intramuscular concentrations of ATP and PCr to subsequent adaptations. It was hypothesized that the reduction of intramuscular concentrations of ATP and PCr would attenuate morphological and metabolic adaptations to functional perturbations such as increased muscular activity and neuromuscular paralysis. Muscle fibre hypertrophy in the plantaris induced by an increased functional demand following synergist ablation was not significantly different between GPA-fed and control rats. In contrast to maintained SDH activity in the control muscles, SDH activity in GPA-fed plantaris decreased following synergist ablation.

Subsequently, total fibre SDH activity was significantly reduced in the hypertrophied GPA-fed plantaris compared to control. Since the enzyme SDH is physically bound to the inner mitochondrial membrane, SDH activity has been used as a marker of mitochondrial content (Hood et al, 1990). An observation of decreased SDH activity combined with increased fibre cross-sectional area can be used as an indication that mitochondrial biogenesis was attenuated when synergist ablation was combined with GPA feeding.

The extent of metabolic and morphological adaptations observed in GPA-fed muscle to TTX-induced paralysis was equal to, or greater than, those seen in control muscle. This observation demonstrates that adaptation to paralysis is not dependent on normal concentrations of ATP and PCr. Furthermore, this study also established that increases in SDH activity subsequent to GPA feeding is dependent on neuromuscular activity. However, increasing neuromuscular activity to supranormal levels (ie. synergist ablation) combined with GPA feeding did not have an additive effect and further augment SDH activity.

In contrast to many acute in vitro studies in which a decrease in ATP concentration subsequently decreased protein synthesis and degradation (Furuno et al., 1990; Medina et al., 1991), protein synthesis was increased in GPA-fed plantaris and tibialis anterior. Furthermore, the rates of protein degradation increased following TTX-induced paralysis in the plantaris and tibialis anterior of both control and GPA-fed rats. Therefore, the response of in vivo protein turnover to ATP depletion is different from in vitro results. Alternatively, the slow progressive decline in PCr and ATP concentrations permits protein turnover mechanisms in skeletal muscle to adapt. Thus, protein turnover responses to acute ATP depletion in vitro may be dissimilar to those following chronic in vivo ATP depletion during GPA feeding. Protein turnover was altered in each muscle following TTX-induced paralysis such that dramatic atrophy occurred. The means by which this was achieved differed between the slow-twitch soleus and the predominantly fast-twitch plantaris and tibialis anterior. Protein synthesis was reduced and protein degradation unaltered in the soleus, whereas protein degradation was increased in both the plantaris and tibialis anterior.

6.1 Review of Thesis Hypotheses

1. Adaptation of single fibres to GPA feeding is muscle and fibre-type specific such that changes will be more pronounced in muscles composed predominantly of type II fibres.
2. The GPA-induced depletion of PCr and ATP attenuates adaptation to synergist ablation and TTX-induced paralysis.
3. Muscle and fibre-type specific adaptation to TTX-induced paralysis occurs based on activation and recruitment patterns of muscles and fibre types. The postural fast-twitch muscle soleus will be affected to the greatest degree and the fast-twitch dorsiflexor tibialis anterior to the least degree.
4. Neuromuscular activity is required for GPA feeding to alter muscle properties towards slow-twitch oxidative metabolism.
5. The rate of protein synthesis is decreased in GPA-fed muscles.
6. GPA feeding increases the rate of protein degradation following TTX-induced paralysis.

6.2 Conclusions

1. In agreement with Hypothesis 1 and 3, the adaptation of fibre morphological and metabolic properties to GPA feeding and TTX-induced paralysis, respectively, was specific to muscle, muscle region and fibre type. In both cases this was most notable in the tibialis anterior. In contrast with the premise of Hypothesis 3, the morphological and metabolic adaptations in the tibialis anterior occurred to a greater extent than in the soleus. Furthermore, superficial type II fibres atrophied more than the more frequently recruited postural type I fibres in the deep region of the tibialis anterior. Therefore, the quantity of activation is less important than the quality of activation in maintaining muscle fibre properties.

2. In contradiction of Hypothesis 2, morphological adaptation to synergist ablation and TTX-induced paralysis was not attenuated in muscle PCr and ATP depleted by GPA feeding. Metabolic adaptation was altered in GPA-fed muscle following both synergist ablation and TTX-induced paralysis. The increase in total SDH activity following synergist ablation was diminished in GPA-fed plantaris relative to control. In contrast, the decrease in SDH activity following TTX-induced paralysis was greater in GPA-fed soleus, plantaris and tibialis anterior relative to control.

3. In corroboration of Hypothesis 4, the alteration of morphological and metabolic properties of muscle towards slow-twitch oxidative following GPA feeding is dependent on neuromuscular activity. The enhanced SDH activity in the soleus, plantaris and tibialis anterior following GPA feeding was abolished when neuromuscular activity was eliminated by TTX. Increased percentage of type I fibres in the soleus and deep region of the tibialis anterior was also eliminated by TTX-induced paralysis. Although in the case of the soleus there was no significant interaction of TTX and GPA main effects on fibre type percentage. A confounding observation was that ATP and PCr concentrations were, in some instances, reduced by TTX-induced paralysis. However, this observation adds credence to the importance of neuromuscular activity. In the case of GPA feeding, the pharmacological reduction of ATP and PCr results in an increased SDH activity. In contrast, when ATP and PCr concentrations are decreased following TTX-induced paralysis SDH activity is reduced and not maintained or increased.

4. In contradiction of Hypothesis 5, in vivo protein synthesis was not decreased following GPA feeding. Rather GPA feeding increased protein synthesis in the plantaris and tibialis anterior and had no effect on the soleus. In contradiction of Hypothesis 6, protein degradation following TTX-induced paralysis was unaffected by GPA feeding.

5. It has recently been published that following GPA feeding maximal mitochondrial ATP synthesis rate is increased (Freyssenet et al., 1994) and energy cost to maintain tension is decreased (Moerland and Kushmerick, 1994). These observations can be attributed to increased activity of mitochondrial aerobic enzymes in addition to a transition in myosin isoforms. These adaptations would be of great relevance to contracting skeletal muscle depleted of phosphocreatine, ATP and reduced creatine kinase activity. As a result of these adaptations GPA-fed muscle depleted of phosphocreatine were able to develop and maintain force similar to control muscles. When functional demand is chronically increased following synergist ablation, GPA-fed muscle which already has increased mitochondrial enzymes, ATP synthesis rate and economy of contraction would seem to be well suited to the increased demand. However, GPA-fed muscle loses SDH activity following synergist ablation. Against practical purposes, it would appear that adaptations to synergist ablation and GPA feeding are incompatible and do not act in harmony. This may be attributable to ATP or PCr concentration attenuating or conflicting with desired muscular adaptations. In contrast, economy of contraction is irrelevant to a paralysed muscle. Thus, adaptations of increased mitochondrial enzyme activity, ATP synthesis rate are also unnecessary. Therefore, it is no surprise that elevated SDH activity in GPA-fed muscle is decreased to a similar level as control muscle following TTX-induced paralysis.

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APPENDIX I: Histochemical Methods

Quantitative Histochemical Methods

The determination of muscle enzyme activity with computer-aided quantitative densitometric analyses of histochemical and cytochemical reaction products in tissue sections is a relatively recent advance in biological sciences (Pette, 1981; Castleman et al., 1984; Blanco et al., 1985). However, in order to be considered quantitative, the histochemical technique must follow stringent guidelines and be able to show precision, specificity, validity and reproducibility (Stoward, 1980). The final reaction product (FRP) arising from a precise technique should be deposited only on the true in vivo subcellular site of the enzyme. A specific technique is one in which the FRP can be attributed to specific activity of the enzyme. A technique can be considered to be valid when the intensity of staining (FRP deposited/cell volume) is related to either the concentration or the specific activity of the enzyme. A reproducible technique is one which produces the same results whoever performs it and irrespective of how often it is performed on equivalent samples. For a detailed description of practical criteria the reader is referred to Stoward (1980).

Perhaps the most important criteria to be met is that the increase in absorbency of the tissue is directly proportional to the enzyme activity or enzyme content of that tissue. Enzyme content and enzyme activity both determine the dehydrogenase reaction intensity and the subsequent absorbency of the muscle fibres in a tissue section. When the tissue section is magnified and its image analyzed by the computer software, the absorbency of each identified fibre is converted to units of optical density (OD) by a logarithmic function that is internally calibrated within the software and hardware of the computer system. When enzyme activity has been expressed as OD, providing that characteristics of the tissue section image were calibrated, the enzyme activity of each fibre has been quantified. Enzyme content is directly proportional to tissue thickness and if calibration and quantification procedures are standardized, section thickness becomes the critical variable in maintaining the relationship between OD and enzyme activity. Tissue thickness has been reported (Martin et al., 1985) to be of the same variance (5%) as that reported in standard biochemical techniques. Therefore, any variance which occurs should be within an acceptable range. The proportionality of increasing absorbance (OD) and enzyme activity is further assured by utilizing both an external and an internal blank correction. The internal blank corrects for tissue thickness, staining, aquamount and glass by subtracting the surrounding area of the slide from the actual absorbance of the tissue section. By determining the OD of the tissue section without substrate the external blank accounts for non-specific staining and diffusion of reaction product within the tissue section, which is then subtracted from the specific staining of tissue sections.

Although Vmax and first order kinetics are not obtained with GPD or SDH histochemical reactions, a steady state reaction condition is. A valid comparison of enzyme activity in different fibre-types and tissue sections is possible because the same conditions exist for each. In fact, enzyme activity in vivo rarely proceeds at a maximal rate because of the existence of both positive and negative modulators. The actual activity of the enzyme is a net balance of both positive and negative influences. When the content of in situ modulators is constant, enzyme activity reaches a steady state at a rate less than Vmax (Spriet, 1987). Therefore, the quantification of enzyme activity in a condition of steady state is valid and can make relative comparisons between different sections of tissue and different fibre-types within a section of tissue.

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Quantitative SDH Assay

Tissue thickness: 10 μm

Phosphate Buffer (100 mM): 6 g NaH_2PO_4
930 mg EDTA
400 ml dH_2O

adjust final pH to 7.6 and bring final volume to 500 ml

Sodium Azide Solution: 65 mg NaN_3
10 ml dH_2O

1-Methoxyphenazine Methylsulphate (mPMS) Solution:
10 mg mPMS
300 μl phosphate buffer

Reaction Medium: with substrate
9.8 ml phosphate buffer
12.3 mg nitroblue tetrazolium
130 mg succinic acid
100 μl mPMS
75 μl NaN_3 solution
check final pH of 7.6

without substrate
same as above minus succinic acid

Procedure:

1. Incubate tissue sections in reaction medium with or without substrate for 8 minutes at room temperature.

2. Air dry in the dark and mount.

Reactions:

$$\text{succinate} + \text{FAD} \longrightarrow \text{fumarate} + \text{FADH}_2$$
$$\text{mPMS} + \text{FADH}_2 \longrightarrow \text{H}_2\text{-mPMS} + \text{FAD}$$
$$\text{NBT} \longrightarrow \text{H}_2\text{-NBT}$$

see GPD for comments.

Quantitative GPD Assay

Tissue thickness: 10 μm

Phosphate Buffer (100 mM): 6 g NaH_2PO_4
400 ml dH_2O

adjust final pH to 7.4 and bring final volume to 500 ml

Sodium Azide Solution: 65 mg NaN_3
10 ml dH_2O

1-Methoxyphenazine Methylsulphate (mPMS) Solution:
2 mg mPMS
300 μl phosphate buffer

Reaction Medium: with substrate

9.8 ml phosphate buffer
9.6 mg nitroblue tetrazolium
20 mg glycerolphosphate
100 μl mPMS
75 μl NaN_3 solution
check final pH of 7.4

without substrate
same as above minus glycerolphosphate

Procedure:

1. Incubate tissue sections in reaction medium with or without substrate for 11 minutes at 37°C.

2. Air dry in the dark and mount.

Reaction:

glyceraldehyde 3-phosphate + $NAD^+ + P_i^{2-} \longrightarrow$
1,3-bisphosphoglycerate + NADH + H^+
mPMS + $FADH_2 \longrightarrow H_2$-mPMS + FAD
NBT $\longrightarrow H_2$-NBT

Comments: The reaction product obtained is a bluish-purple NBT diformazan which has a peak absorbance wavelength of 550 nm. Absorbance of light by any NBT monoformazan reaction product was prevented by a cutoff filter which eliminated light with a wavelength less than 450 nm. The addition of mPMS acts as an exogenous electron carrier which transfers reducing equivalents (NADH, NADPH), generated by the dehydrogenase, which then reduce NBT to form the bluish-purple diformazan from the yellow NBT. In the absence of mPMS, the reaction pattern is indicative of the flavoprotein reductase and not the primary dehydrogenase (Van Noorden and Butcher, 1984). The transport of H^+ to oxygen must be inhibited in order to measure dehydrogenase activity therefore, azide is included in the reaction mixture because it inhibits cytochrome oxidase in the electron transport chain (Van Noorden and Tas, 1984). In contrast to succinate dehydrogenase which is bound to the mitochondrial membrane, the α-glycerophosphate dehydrogenase enzyme measured here is cytosolic. As a cytosolic enzyme it is susceptible to diffusion from its cytosolic location and can be lost in the incubation buffer. It is assumed that because samples from different treatments were incubated together loss of enzyme is proportional between samples and is a constant variable in each incubation.

Qualitative Myofibrillar ATPase Assay

Tissue thickness: 10 μm

Pre-Incubation AMP Buffer:

1.9 ml AMP
400 mg NaN_3
4 g $CaCl_2$
300 ml dH_2O

adjust final pH to 9.4 and bring final volume to 400 ml with additional dH_2O.

Incubation AMP Buffer:

4.8 ml AMP
500 mg NaN_3
400 ml H_2O

adjust final pH to 9.8 then bring to a final volume of 500 ml with additional dH_2O.

Reaction Medium (Fresh):

10 ml AMP buffer
80 mg $CaCl_2$
12 mg ATP

check final pH is still at 9.8 and pre-warm to 37°C.

Procedure:

1. Incubate tissue sections at room temperature in 10 ml pre-incubation buffer in a reaction jar. Reaction time 10 minutes. Wash 2-3 times with dH_2O.

2. Add reaction medium to reaction jar and run reaction for 18-20 minutes in water bath of 37°C. Wash with dH_2O 2-3 times.

3. 2% $CoCl_2$ (200 mg in 10 ml dH_2O) at room temperature for 10 minutes. Wash 2-3 times.

4. 1% $(NH_4)_2S$ (100 μl in 1 ml dH_2O) at room temperature for 40 seconds. Wash 2-3 times.

5. Dry and mount slides

Comments: Through the enzyme activity of actomyosin ATPase phosphate ions are released from the hydrolysis of ATP. A black-staining reaction product occurs when the sulfate ions react with the cobalt ions bound to the phosphate ions to produce cobalt sulfate, a black compound. The abbreviated biochemical reactions involved are:

$$ATP \longleftrightarrow ADP + PO_4^+$$

$$CoCl_2 + PO_4^+ \longleftrightarrow Co(PO_4)_2 + 2HCl$$

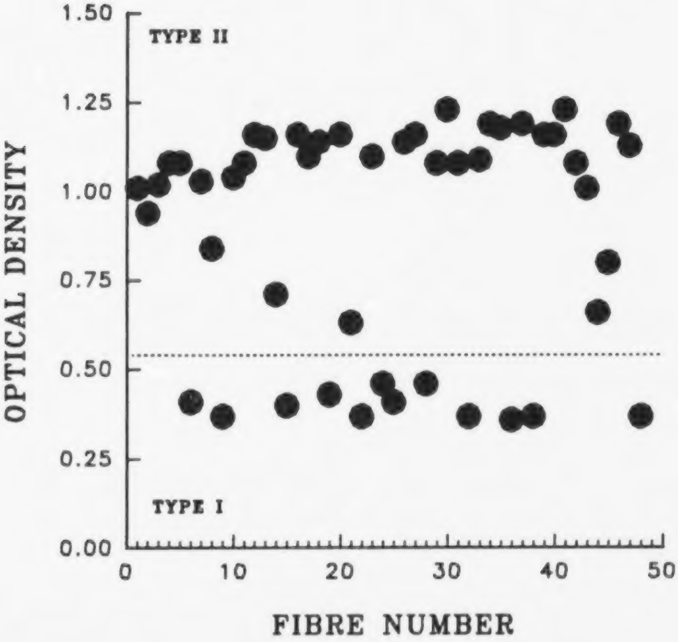
$$Co(PO_4)_2 + (NH_4)_2SO_4 \longleftrightarrow CoSO_4 + 2NH_4PO_4$$

When the ATPase inhibitors ouabain, oligomycin and calmidizolium (R23471) are added to block Na^+/K^+-ATPase and SR Ca^{2+}-ATpase the reaction is specific for actomyosin ATPase.

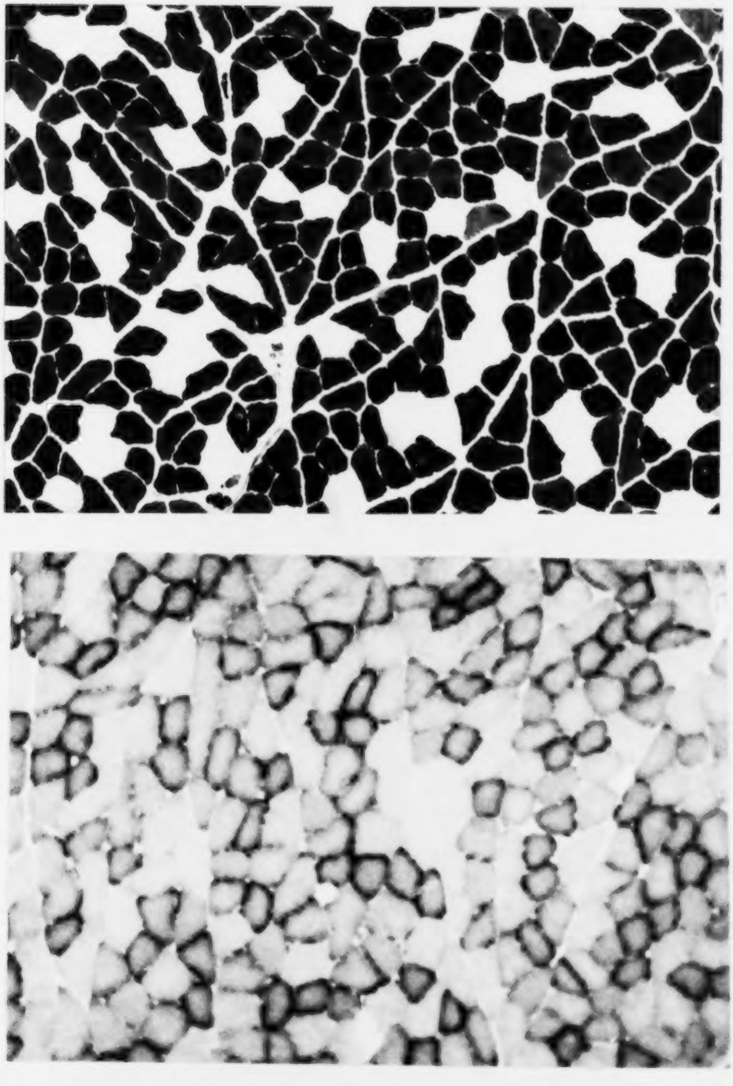
Representative Micrographs and Single Fibre Data

The quantitative histochemical staining in the chapters of this thesis has been analyzed and the enzyme activity expressed as optical density per single fibre. The fibres are then classified as type I or II single fibre data based on intensity of myofibrillar ATPase staining. In each muscle section selected for analysis an assortment of type I and II fibres are present. With alkaline preincubation, dark staining fibres were classified as type II and light staining fibres type I. The classification procedure was not done subjectively, but was performed on a muscle by muscle basis by plotting fibre number vs. optical density as demonstrated in Figure VII-1. Fibres with an ATPase optical density less than 0.50 have been classified as Type I. Raw cross-sectional area (CSA) and optical density values of ATPase, GPD and SDH of individual muscle fibres are tabulated in Table VII-1. The units for GPD and SDH reactions are optical density (OD) per minute of reaction time ([OD/min] x10^4).

FIGURE VII-1. Representative optical density values for plantaris single fibres. Dashed line indicates cut off for type I and II fibres.



FIBRE #	CSA	ATP	GPD	SDH
1	1112.38	1.01	7.18	32.75
2	1109.58	0.94	4.45	83.17
3	1081.52	1.02	8.39	33.17
4	1611.76	1.08	10.52	79.83
5	1033.83	1.08	10.52	65.67
6	1308.77	0.41	3.24	43.58
7	1557.05	1.03	11.42	55.67
8	1467.28	0.84	4.15	65.67
9	2164.45	0.37	3.55	48.17
10	1488.32	1.04	9.91	56.92
11	1350.85	1.08	5.06	71.08
12	1328.41	1.16	7.48	79.83
13	1335.42	1.15	6.27	81.08
14	1764.66	0.71	3.85	44.83
15	2327.17	0.40	3.55	49.83
16	1139.03	1.16	5.67	75.25
17	1621.58	1.10	10.82	54.00
18	1280.71	1.14	10.21	56.92
19	1594.93	0.43	3.55	44.83
20	1449.04	1.16	9.61	70.25
21	1204.96	0.63	4.15	58.17
22	2976.64	0.37	3.24	50.25
23	859.89	1.10	9.00	34.00
24	2119.56	0.46	3.55	55.25
25	1799.73	0.41	3.24	61.92
26	1117.99	1.14	9.00	75.25
27	864.10	1.16	9.30	33.17
28	1708.55	0.46	3.85	51.08
29	862.69	1.08	3.85	78.58
30	1070.30	1.23	6.88	78.58
31	1117.99	1.08	6.58	76.50
32	2473.05	0.37	2.94	44.42
33	1154.46	1.09	6.27	80.25
34	1655.25	1.19	11.12	59.42
35	1315.78	1.18	10.52	59.00
36	1980.69	0.36	2.64	46.50
37	433.45	1.19	6.27	87.75
38	3066.42	0.37	2.94	45.67
39	1103.97	1.16	11.42	58.17
40	1026.81	1.16	10.82	40.25
41	1165.69	1.23	10.21	36.92
42	1176.91	1.08	4.45	79.83
43	1188.13	1.01	4.45	69.83
44	956.68	0.66	3.24	74.00
45	792.55	0.80	3.85	68.17
46	1176.91	1.19	8.70	40.67
47	715.40	1.13	5.67	86.50
48	2576.86	0.37	2.94	44.42



APPENDIX II: Protein Turnover Methodology

Protein Synthesis

In vitro and in vivo skeletal muscle protein synthesis is invariably determined by the incorporation of isotopically labelled amino acids into protein. Although in vitro methods can be simple and inexpensive, the validity of results are questionable due to the negative nitrogen balance of muscles removed from in vivo humoral and physical influences, and the presence of hypoxic cores within even small muscles (Sugden and Fuller, 1991). Furthermore, transport limitations and preferential charging of the aminoacyl-tRNA pool with amino acids from intracellular protein degradation may require monitoring specific radioactivity throughout the experiment which is difficult. There are two methods that are widely used for measuring in vivo protein synthesis; the constant infusion technique and the flooding dose technique. In the constant infusion technique, a tracer quantity of isotope is infused intravenously over a matter of hours to isotopically equilibrate the plasma amino acid pools. The flooding dose technique uses a large quantity of isotope, rapidly administered intravenously or intraperitoneally to flood the precursor pools and equilibrate them. Unless the complicated and difficult task of measuring the direct precursor for protein synthesis, aminoacyl-tRNA, is performed, both methods use measurements of tissue and/or plasma free amino acid specific activity instead and assume they are equal. Despite their widespread use, there are criticisms of each method. The constant infusion method is much longer and more difficult technically than the flooding dose technique. In order for specific radioactivity to reach equilibration in less than an hour, a priming dose of isotope must be given at the start of infusion. During prolonged perfusion, isotope reutilization can present a considerable problem (Melville et al., 1989) and it is difficult to identify the precursor pool to use to calculate protein synthesis as the intracellular and plasm specific activities can be sufficiently different (McNurlan et al., 1979, Pomposelli et al., 1985). Furthermore, constant restraint of the animals for the duration of the experiment and associated stress can influence rates of protein synthesis.

The flooding-dose method improves on some of these limitations because the combination of a large amount of isotope and a larger amount of unlabelled amino acid results in a rapid equilibration of specific activities in the aminoacyl-tRNA, intracellular, and plasma pools (Garlick et al., 1980b). Furthermore, the short labelling period (10-15 min) limits the recycling of amino acid tracer and the plasma and tissue specific activities remain almost constant during this period of incorporation (Garlick et al., 1980b; Reeds et al., 1982; Jepson et al. 1986). When the rate of protein synthesis is being determined on a mixture of protein with a spectrum of turnover rates, as is present in skeletal muscle, protein synthesis is consistently underestimated by the constant infusion method but is accurately reflected by the shorter duration flooding dose method (Samarel, 1991). Furthermore, the flooding-dose animal is free to move about its cage during the incorporation period and is relatively free of undue stress. However, the flooding dose technique is not without its limitations. The aminoacyl-tRNA pool may not equilibrate simultaneously with the tissue and plasma pools, but may lag behind by a few minutes (Robinson and Samarel, 1990). Combined with the rapid decline of plasma specific activity limiting the useful labelling period to 10 minutes, a lag of two minutes represents a significant proportion of the labelling period and may result in an underestimation of protein synthesis (Robinson and Samarel, 1990). Protein synthesis during the short incorporation period may be disproportionately influenced by short-lived proteins and give significantly higher estimates of protein synthesis (Hasselgren et al., 1988). A concern of the flooding dose method is that the large amount of amino acid injected may influence protein turnover. Garlick et al. (1980) concluded that phenylalanine was preferable to leucine because it was more soluble, less expensive, the free pool of phenylalanine is small and is easily flooded and the tissue radioactivities in tissue and plasma were closer than with leucine. Furthermore, no effect of phenylalanine on protein turnover has been shown. The flooding dose technique is technically simpler, avoids the need for prolonged restraint and is generally preferred to the constant infusion technique as a method to measure protein synthesis. The simplicity of the method and an increase in the incorporation time to 15-20 minutes is achieved when an intraperitoneal instead of intravenous injection is used (Jepson et al., 1986)

Protein Degradation

In vitro methods to estimate protein degradation involve determination of the rate of release of tyrosine or phenylalanine from protein. Muscles are incubated in the presence of cycloheximide, which prevents re-incorporation of amino acids into protein (Tischler et al., 1982). However, the validity of in vitro data obtained from incubated skeletal muscle must be questioned for the same reasons as in protein synthesis. In vivo protein degradation is generally measured indirectly as the difference between the rate of accumulation of protein, or growth, and the rate of protein synthesis. The criticism of this method is that synthesis is measured over minutes, or hours at most, and it takes measurement over days to determine the small change in growth. Furthermore, the calculation depends on the assumption that the rate of protein synthesis is constant over the period of time that growth is determined (Sugden and Fuller, 1991). Improvements in the gross measurement of body mass are made by determining the protein mass or mass of a specific amino acid of each tissue.

Detailed Procedures:
Measurement of in vivo protein turnover

Fractional rate of protein synthesis

1. Injection of flooding dose of (^{3}H) Phenylalanine (Phe)

Solutions.

1. Phosphate buffered saline (PBS) pH 7.4

To make 2 L:

16.9 g Nacl
5.1 g $Na_2HPO_4.H_2O$
0.28 g NaH_2PO_4

- dissolve reagents in 1900 mL MQ water
- adjust to pH 7.4 with 10 NaOh
- bring volume to 2 L with Milli Q (MQ) water
  autoclave 20 min.

2. (^{3}H) Phe solution

The final solution will contain 50 μCI/100 g body wieght and 150 umol Phe/100 g body wieght, contained in 1.5 mL PBS/100 g body weight. Make - 10% more solution than needed to account for dead space in syringes. This solution should mot be made more than 1 to 2 days in advance.

2.065 g Phe ($C_9H_{11}NO_2$
PBS
(^{3}H) Phe (1mCi/mL)

- dissolve Phe in 100 mL PBS
- this solution will take - 1 hour to dissolve

- draw out from isotope stock bottle 50 uL (^{3}H) Phe per 100 g body weight, using a tuberculin syringe
- inject into an uncoated, sterile, and stoppered 10 - 15 mL vacutainer tube
- inject 1.5 mL of Phe solution per 100 g body weight through a 0.2 μ Millipore filter into the vacutainer tube
- mix and refrigerate

Procedure:

On the day of the experiment, animals were weighed and syringes of radioactive solution prepared, One rat was injected every 7 minutes. After precisely 20 minutes, the rat was stunned by immersion in CO_2 gas and then killed by cervical dislocation. The rat was immersed in ice water and the skin removed from the hindlimbs. This will ensure that protein turnover is halted. The carcass was removed from the ice bath after not less than one minute and the tibialis anterior (TA), plantaris (PL) and soleus (SOL) muscles were removed bilaterally and frozen in liquid nitrogen. The fractional rate of protein synthesis was determined for each muscle. Tissues were stored at -50°C.

2. Tissue homogenization

The objective of tissue homogenization was to separate free from protein-bound amino acids prior to determination of their specific radioactivities.

Solutions

1. 2% Perchloric acid ($HClO_4$)

To make 2 L:

- 57 mL 70% $HClO_4$
- bring volume to 2 L with distilled water

Procedure:

Tissues were homogenized using a motorized ground glass tissue homogenizer in ice cold 2% $HClO_4$. Volumes of 2.0 ml, 1.0 ml and 0.75 ml was used for the TA, PL and SOL, respectively. The homogenate was pipetted into a 15 mL screw capped Pyrex test tube. The homogenizer and pipette was rinsed 2 times with 1.0 mL 2% $HClO_4$ and washings combined with the homogenate. Homogenates were stored on ice, until centrifuged. The homogenate was centrifuged at 1000 x g for 15 min. The supernatant was decanted into a 10 ml test tube and saved for analysis of intracellular Phe. The pellet was saved for analysis of protein-bound Phe.

3. Intracellular fraction

The objective of this step was to precipitate perchlorate ions and neutralize the solution for subsequent enzymic conversion.

Solutions

1. Saturated potassium citrate ($K_3C_6H_5O_7$)

   To Make 40 mL:

   - 70 g $K_3C_6H_5O_7$
   - dissolve in 40 mL distilled water
   - keep adding 5 g of potassium citrate until no more will dissolve
   - gently heat until dissolved
   - cool

Procedure:

Half a volume of saturated potassium citrate was added to the intracellular fraction. A whitish crystalline precipitate formed. Samples were contrifuged at 2000 x g. The supernatant (intracellular fraction) was decanted into 10 mL test tubes and stored frozen. The precipitate was discarded.

4. Protein-bound fraction

The purpose of this step was to remove any free amino acids, then hydrolyse the protein-bound amino acids, remove the acid, and neutralize the solution for subsequent enzymic conversion.

Solutions:

1. 2% $HClO_4$ (see above)

2. 6 N HCl

To make 1 L:

500 mL concentrated HCl
500 mL distilled water

- pour acid into water
- cool
- bubbble with nitrogen gas (10 - 15 minutes)

3. 0.5 M Tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) (pH 6.3)

To make 100 mL:

14.71 g tri-sodium citrate

- dissolve in 100 mL distilled water
- adjust to pH 6.3 with 10 M HCl

Procedure:

The protein pellet was washed with 8 mL ice-cold 2% $HClO_4$ and centrifuged for 15 min at 3000 x g. The supernatant was discarded. This step was repeated twice. Five mL 6 N HCl was added. It was important to make sure that the Pyrex test tubes had no chips on lip (to prevent evaporation). Tubes were purged with nitrogen, capped with screw caps with teflon liners, and heated to 110^{0}C for 24 hours. After 30 min., caps were tightened, checked for evaporation, and tubes were vortexed. The solution turned black, When there was evaporation, lost volume was replaced with 6 N HCl.
Acid was then removed. It may be removed by freeze drying, nitrogen gas, or vacuum centrifuge (Speed-vac concentrator). Vacuum centrifugation at 65^{0}C was preferred. The amino acids were resuspended in 0.5 mL sodium citrate (pH 6.3). The pH was adjusted to 6 to 7 using 10 N NaOH and litmus paper

and stored frozen. This solution is ready for enzymic conversion.

5. Enzyme conversion

The objective of this step was to convert Phe to beta- phenylethylamine (PEA) so that it could be separated from other amino acids, such as tyrosine and tyramine, that may have become labelled during the period of incorporation.

Solutions:

1. 0.5 M sodium citrate (pH 6.3) (see above)

2. Enzyme suspension

This contains 2 Units/mL L-tyrosine decarboxylase (Sigma T4379), 1 mg/mL pyridoxal phosphate (Sigma P9255) in 0.5 mL 0.5 M sodium citrate (pH 6.3). Tyrosine decarboxylase can be used in place of Phe decarboxylase, because it is contaminated with the former. It is generally less costly than Phe decarboxylase.

To make 50 mL of enzyme suspension:

100 units tyrosine decarboxylase
0.05 g pyridoxal phosphate
0.5 M sodium citrate buffer (pH 6.3)

- combine ingredients
- the enzyme dose not dissolve
- the suspension may be stored at -50°C
- mix well before using

Procedure:

Half a mL of enzyme suspension was added to 1.0 mL of supernatant fraction or 0.5 mL of neutralized hydrolysate. The tubes were stoppered and incubated for 17 h in a shaking water bath at 50°C.

6. Solvent extraction

The purpose of this step was to separate the PEA from other amino acids. PEA was extracted by the method of Suzuki and Yagi (1976) as modified by McCallister (1987).

Solutions

1. 3 M NaOH

To make 100 mL:

12 g NaOH
- dissolve in 100 mL distilled water

0.1 N H_2SO_4

To make 1 L:

2.8 mL concentrated sulphuric acid
- add to 1 L distilled water

3. Chloroform:n-heptane (1:3 v/v)

Use distilled reagent grade chloroform and heptane. Do not use HPLC grade chloroform.

Procedure:

Half a mL of 3 M NaOH was added to the entire incubated enzyme solution. Amino acids become basic and polar; PEA becomes neutral and non-polar. Five mL chloroform-n-heptane was then added. Tubes were stoppered, shaken, and then centifuged at 500 g for 5 min. Amino acids, because they are polar remain in the aqueous phase; PEA because it is non-polar is extracted into the organic phase. The upper organic phase was carefully removed by pipette. Care was taken not to contaminate it with any of the bottom aqueous phase. Chloroform is more dense than water; the organic layer is on top because n-heptane, which is miscible in chloroform, makes the organic solution less dense. The aqueous phase was discarded. Five mL chloroform and 2 mL 0.1 N H_2SO_4 were added to the organic phase. Tubes were stoppered, shaken, and then centrifuged at 500 g for 5 min. Under acidic conditions PEA is polar and is extracted into the aqueous phase. The top aqueous phase was removed by pipette. The organic layer settles to the bottom because the added chloroform makes the organic solution more dense. The organic layer was discarded. The 0.1 N H_2SO_4 containing PEA was left uncovered at room temperature to ensure all traces of chloroform were removed. Residual chloroform caused poor pipetting. Once pipetted, solutions were tightly covered.

7. HPLC

The purpose of this step was to measure the concentration of PEA in the final aqueous extract.

Solutions:

All solutions were made up in glassware that was rinsed with HPLC grade water. Chemicals were always weighed on the same balance. Pipettes were calibrated before use. Great care was taken when pipetting.

1. 5 μmol/mL Ethanolamine (EA) ($C_2H_7NO.HCl$)

To make 200 mL:

0.0975 g EA

- dissolve EA in 200 mL HPLC water
- this stock solution may be used for 3 d

To make internal standard solution (25 nmol/mL)

- combine exactly 0.500 mL of stock solution with 100 mL HPLC grade water
- the working solution is made daily

2. 5 μmol/mL beta-Phenylethylamine ($C_8H_{11}N.HCl$) (PEA)

To make 200 mL:

- dissolve PEA in 200 mL HPLC grade water
- refrigerate
- this stock solution (5 umol/mL) may be used for 3 d

To make internal standard (25 nmol/ml):

- combine exactly 0.500 mL of stock solution with 100 mL HPLC grade water

- the working solution is made daily